UPTAKE AND PROCESSING OF CHLOROPLAST POLYPEPTIDES

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By

Colin Robinson

B.Sc. (Edinburgh)

Department of Biological Sciences

University of Warwick

Coventry

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SUMMARY

A chloroplast protease capable of processing the precursor of ribulose bisphosphate carboxylase small subunit to the mature size has been purified 350-fold from stromal extracts of pea leaves. The enzyme has a molecular weight of about 180,000 daltons, a pH optimum near 9.0, and is inhibited by metal-chelators but not by serine- or thiol-protease inhibitors. The partially purified enzyme is also capable of processing the precursors of wheat and barley pre-plastocyanin to the mature size, and is therefore neither precursor- nor species-specific. The enzyme displays a high degree of reaction specificity in that it has failed to cleave all protein substrates tested other than precursors destined for the chloroplast.

The small subunit precursor (molecular weight 20,000) is processed to the mature size (molecular weight 14,000) via an intermediate of molecular weight 18,000. The second cleavage can be inhibited by pre-incubation of the precursor with iodoacetate.

A preliminary investigation into the basis for the specificity of the small subunit precursor processing reaction has been carried out. Proline, lysine and arginine residues in the small subunit precursor polypeptide chain have been replaced by amino acid analogues of these residues. The abnormal precursors are very poor substrates for the purified processing enzyme, and are imported into intact isolated chloroplasts at much-reduced rates. The significance of these observations is discussed with reference to the primary structure of the small subunit precursor.

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DECLARATION

The work contained in this thesis was the result of original research conducted by myself under the supervision of Professor R. J. Ellis. All sources of information have been acknowledged by means of reference. None of the work has been used in any previous application for a degree.

Colin Robinson

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ABBREVIATIONS

A	absorbance
ATP	adenosine triphosphate
cDNA	complementary DNA
CF_1	coupling factor 1
Ci	Curie (3.7×10^{10} disingtegrations per second)
cpm	counts per minute
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethyleneglycol-bis (-amino ethyl ether) N,N'-tetra acetic acid
Fe-S	Iron-sulphur
GTP	guanosine triphosphate
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
KD	kilodaltons
mA	milliampere
mRNA	messenger RNA
m.wt.	molecular weight
NBM	nitrobenzyloxymethyl
NBPC	N-(3-nitrobenzyloxymethyl)-pyridinium chloride
NP40	Nonidet P40
oligo(dT)	oligo-deoxythymidylic acid
P20	precursor to the small subunit of RuBPCase
PMSF	phenylmethylsulphonyl fluoride

poly(A)	poly-adenylic acid
POPOP	1,4-bis-(5-phenyloxazol-2-yl)benzene
PPO	2,5-phenyl oxazole
RNA	ribonucleic acid
RuBPCase	ribulose-1,5-bisphosphate carboxylase
SDS	sodium dodecyl sulphate
SSC	standard slaine citrate
SSU	small subunit of RuBPCase
TCA	trichloroacetic acid
TEMED	N,N,N,'-tetramethylene diamine
Tris	2-amino-2-hydroxymethyl propane-1,3-diol
tRNA	transfer RNA

SECTION I - LITERATURE REVIEW

1. INTRODUCTION

The experiments described in this thesis were designed with the aim of characterising the mechanism by which cytoplasmically-synthesised proteins are transported into chloroplasts. The literature review is intended to provide a comprehensive background against which the data can be evaluated in terms of the validity of the experimental approaches employed, and the significance of the results obtained.

The structure, function and biogenesis of chloroplasts is reviewed in Section I.3, with particular emphasis being placed on studies of the sites of synthesis of chloroplast proteins.

The subject of intracellular protein transport is discussed in Section I.4, with the description of available data on protein transport from several systems in addition to chloroplast protein transport. A vast amount of literature is available on the transport of eukaryotic and bacterial secretory proteins, and on the transport of proteins into mitochondria. In comparison, the research area of chloroplast protein transport is still in its infancy, and hence data obtained in this study can be usefully compared with data already available from analogous experiments on protein transport in other systems.

In many protein transport systems, including that of chloroplasts, the translocation of the polypeptide is often accompanied by, or followed by, proteolytic processing of the polypeptide to yield the mature size. The main aim of the experiments described in this thesis has been to

characterise the chloroplast proteolytic activity responsible for the processing of imported polypeptides. Section I.4 contains a survey of a variety of protease classes so that features of the chloroplast processing activity can be compared with those of other proteases.

The aims of the experiments described in this thesis, and the experimental approaches adopted, are described in Section I.5.

2. CHLOROPLAST STRUCTURE, FUNCTION AND BIOGENESIS

A. Chloroplast structure and function

Chloroplasts are the most well-known of the family of eukaryotic plant organelles known as plastids (Kirk and Tilney-Bassett, 1978). As mediators of photosynthesis, chloroplasts are responsible for the fixation of carbon dioxide and it is because of this crucial role that they have been intensively studied. However, chloroplasts carry out a number of other metabolic functions in plant cells, including the synthesis of numerous sugars, amino acids, nucleic acids, pigments and complex lipids. Furthermore, chloroplasts possess their own DNA, RNA, and protein-synthesising machinery and therefore contribute to the plastid genotype (discussed in detail in Section I.2F). During cell division and differentiation, the developing chloroplasts also divide, accompanied by the replication of chloroplast DNA (Rose *et al.*, 1975; Boffey *et al.*, 1979).

Chloroplasts are typically lens-shaped, with a diameter of about 5 μm and a length of about 10 μm. The number of chloroplasts per cell varies from one species to another; *Zea mays* has about 30 chloroplasts per mature leaf cell, some varieties of wheat have 200, while the alga *Chlamydomonas reinhardii* has a single, large chloroplast per cell.

Structurally, the chloroplast consists of an *envelope* (of two membranes) enclosing a hydrophilic *stromal* phase, within which lies the photosynthetic (*thylakoid*) membrane network. Each of these three fractions will be considered in greater detail below.

B. The Chloroplast envelope

The envelope consists of two distinct membranes separated by an intermembrane space. The outer membrane is freely permeable to small molecules, but the inner membrane shows very specific permeability properties and contains a number of specific translocation systems (Heldt, 1976; Flugge & Benz, 1984). In addition, a number of enzyme activities are associated with the envelope, including acyl-CoA synthetase, phosphatidic acid phosphatase, galactosyl transferase and adenylate kinase (Douce *et al.*, 1973; Joyard and Douce, 1979; Murakami and Strotmann, 1978). Biochemical studies on the separated membranes have demonstrated that each membrane has its own characteristic polypeptide and lipid composition (Block *et al.*, 1983a, b). Electrophoretic analysis of envelope polypeptides indicates the presence of numerous proteins, though the envelope membranes represent less than 1% of total chloroplast protein.

C. The Chloroplast stroma

The soluble stromal phase of the chloroplast contains a large number of enzymes responsible for a variety of metabolic activities as described in Section I.2A. The stroma also contains DNA, RNA, ribosomes and all the other components involved in protein synthesis (Boulter *et al.*, 1972).

In most chloroplasts, the enzyme ribulose bisphosphate carboxylase-oxygenase (RuBPCase) accounts for at least 50% of soluble leaf protein. This enzyme will be considered in more detail later, since the synthesis and assembly of this protein are central to this thesis.

It is important to realise that the term "stromal", as used in the remainder of this thesis, is defined operationally as the soluble phase released by lysis of intact chloroplasts. *In vivo* it is likely that in the intact chloroplast, a number of "stromal" enzymes are loosely associated with either the thylakoid membrane or the inside of the inner envelope membrane.

D. The thylakoid membrane

The chlorophyll-containing membranes of the thylakoid network are the site of the light-harvesting and energy-transduction reactions of photosynthesis. The network is usually considered to consist of a number of stacks of membrane-bound vesicles (the grana) which are interconnected by a matrix of protrusions arising from the granal stacks (Kirk and Tilney-Bassett, 1978). Grana vary greatly in shape and dimensions in different photosynthetic tissues that have been studied.

The thylakoid membranes consist of approximately 50% (by weight) protein and 50% lipid. The protein complement is primarily involved in harvesting of light and in energy transduction. Light is absorbed by two distinct protein-chlorophyll assemblies termed photosystems I and II. The majority of chlorophyll molecules in each photosystem function as light-harvesting "antennae" molecules which channel excitation energy to a small number of "reaction centre" chlorophyll molecules (Anderson, 1975; Thornber, 1976). In the reaction centres, the excitation energy is transmitted by means of an electron to an acceptor molecule. Two sequences of electron carriers of increasingly positive oxidation-reduction potential carry electrons away from the reduced acceptors of the photosystems and are thought to interconnect in the "Z-scheme", originally proposed by Hill and Bendall (1960). The flow of electrons through the carriers is believed to create an electrochemical potential difference of protons, with the intrathylakoidal space becoming acidified. An ATP-synthetase complex couples the diffusion of protons back through the membrane with ATP synthesis. The coupling factor (CF) of the ATP synthetase is located on the stromal side of the thylakoid

membrane (Bohme, 1978). The complex contains, in addition, a membrane-bound portion (CF_o).

E. Ribulose bisphosphate carboxylase-oxygenase (RuBPCase)

RuBPCase catalyses the first of the series of reactions whereby CO_2 is fixed in the plant leaf and converted to carboxhydrates and other organic compounds. This key reaction can be summarised as follows:

D-ribulose-1,5-bisphosphate + CO_2 + H_2O -->

3-phosphoglycerate (2 molecules)

The enzyme constitutes the major soluble leaf protein in plants, and is by far the major soluble protein of chloroplasts where it may represent as much as 90% of total soluble protein. The enzyme was first purified from spinach leaves by Weissbach et al. (1956). Subsequent studies have shown that the enzyme also catalyses the oxygenation of ribulose-1,5-bisphosphate (Bowes et al., 1971):

D-ribulose-1,5-bisphosphate + O_2 -->

3-phosphoglycerate + 2-phosphoglycolate.

Phosphoglycolate so produced enters a series of reactions that result in the evolution of CO_2, a metabolic activity known as photorespiration. The oxygenase activity of RuBPCase has been the subject of much study since it reduces the net assimilation of CO_2 by photosynthesis, thereby decreasing plant growth and crop yield. Under natural conditions it is thought that ratio of carboxylation to oxygenation in vivo is between 3:1 and 4:1 (Farquhar et al., 1980). The function of photorespiration is still obscure but all RuBPCases so far studied have been found to exhibit oxygenase activity, even those present in some anaerobic bacteria.

RuBPCase has been purified from a wide variety of plant, algal and bacterial souces (Siegelman and Hind, Eds., 1978); McFadden, 1980). All of the plant and algal enzymes studied to date have a molecular weight of approximately 550,000 and are composed of eight large subunits (52-56,000 molecular weight) and eight small subunits (12-16,000 molecular weight). The molecular weight and subunit composition of bacterial enzymes are more variable. The above L_8S_8 form has been reported, but a variety of other forms, including L_2, L_4, L_6, L_6S_6 and L_8, are also believed to exist (for review see McFadden, 1980).

In all of the above enzyme forms, the large subunit catalyses both carboxylation and oxygenation reactions, possibly by means of a common active site (Badger and Lorimer, 1976; Brown et al., 1980). The activation of the enzyme by binding of CO_2 (at a site other than the active centre) is also believed to be mediated by the large subunit (Lorimer, 1981).

The function of the small subunit is not known, though Andrews and Ballment (1983) have shown that the presence of small subunits in the Synchococcus holoenzyme is essential for catalysis. It has been suggested that the small subunit has a role in the regulation or activation of the enzyme activity, but there is no clear evidence to support this possibility.

F. SYNTHESIS OF CHLOROPLAST PROTEINS

(i) Chloroplast DNA

The existence of an autonomous genetic system in chloroplasts has been inferred from plant breeding experiments dating from the early years of this century. Certain plastid defects are inherited in such a way as to suggest that the abnormal genes reside in the plastid rather than in the nucleus (reviewed in Kirk and Tilney-Bassett, 1978). However, it was not until the 1960s that the presence of DNA in chloroplasts was established beyond doubt. The first direct evidence for the existence of chloroplast DNA came from electron microscopy studies on cells of Chlamydomonas moewusii (Ris and Plaut, 1962). Shortly afterwards, Kirk (1963a, b) showed that DNA isolated from chloroplasts of Vicia faba differed in base composition (guanosine/cytosine ratio) from the DNA of isolated V. faba nuclei. Since these reports, the base characteristics of chloroplast DNAs from a wide range of plants have been reported (listed in Kirk and Tilney-Bassett, 1978). The chloroplast DNAs show very similar values for base composition and buoyant density, whereas those of the nuclear DNAs from the same range of species vary considerably.

A further distinguishing feature of chloroplast DNAs is that they lack 5-methylcytosine (as do prokaryotic DNAs), whereas a proportion of the cytosine residues of higher plant nuclear DNAs is invariably methylated (Braverman & Eisenstadt, 1964; Herrmann, 1972; Wells & Birnstiel, 1969; Whitfield & Spencer, 1968; Shah & Levings, 1973; Tewari & Wildman, 1966).

Electron microscopy studies of a variety of chloroplast DNAs have shown that the DNA exists as closed circular molecules with a contour length of about 45 μm in higher plants (Manning & Richards, 1972; Kolodner & Tewari, 1975). Using sedimentation equilibrium measurements on the closed circular DNA preparations, Kolodner et al. (1976) obtained values of 89.1, 98.2 and 97.2 million daltons for the molecular weights of chloroplast DNA from pea, lettuce and spinach, respectively. Each chloroplast in high plants or algae contains many copies of the chromosome: a typical mature leaf chloroplast contains 20-50 DNA molecules (Kung & Williams, 1969).

Studies on the kinetic complexity of the chloroplast DNA molecule have indicated that there is little or no reiteration of sequences in the molecule (Bedbrook and Kolodner, 1979). However, chloroplast DNA from several species has been shown to contain a large section, about 20 kilobase pairs long (15 million daltons), which is present twice in the chromosome in an inverted orientation. This inverted repeat has been shown to contain the genes coding for the 16S, 23S and 5S plastid ribosomal RNAs (Bedbrook et al., 1977).

Allowing for known repeats on the plastid chromosome, and the presence of a number of transfer RNA genes (Haff and Bogorad, 1976; Steinmetz et al., 1978) the length of the plastid DNA molecule is such that it could encode about 120 proteins, each of 40 KD. The number of genes identified on the chloroplast chromosome is increasing steadily, and includes those encoding about 50% of the thylakoid proteins (Bottomley & Bohnert, 1982).

(ii) The intracellular origin of chloroplast proteins

As well as containing DNA, chloroplasts possess all the machinery required for protein synthesis, including ribosomes, tRNAs, mRNAs, aminoacyl-tRNA synthetases, initiation factors and elongation factors (reviewed in Bohnert et al., 1982). As described in the previous section, evidence for the synthesis of proteins by chloroplasts has been obtained from genetic studies dating from the early part of this century: a number of mutations affecting chloroplasts are inherited in a non-Mendelian fashion and are thus believed to reside in the plastid genome. However, the majority of mutations affecting chloroplasts have been found to follow Mendelian rules of inheritance, suggesting that the biogenesis of chloroplasts requires the activities of both nuclear and chloroplast genetic systems.

Further evidence for the contribution of nuclear genes to chloroplast development has been obtained from mutants which lack chloroplast ribosomes. Feierabend and Wildner (1978) have shown that the leaves of rye seedlings grown at 32^{o}C lack chloroplast ribosomes but contain many of the usual proteins (including the small subunit of RuBPCase), suggesting that these proteins must be synthesised on cytoplasmic ribosomes. Proteins that are absent in the leaves of such plants include the large subunit of RuBP carboxylase, the alpha and beta subunits of the ATPase coupling factor, and several cytochromes and thylakoid polypeptides.

Another approach employed to study the synthesis of nuclear- and chloroplast-encoded chloroplast polypeptides has been the use of

selective inhibitors of cytoplasmic or chloroplast protein synthesis. Chloroplast ribosomes are very similar to the prokaryotic type, (70S rather than the 80S eukaryotic type), and show similar antibiotic sensitivities (Boulter *et al.*, 1972). A number of experiments have involved the selective use of an inhibitor of chloroplast ribosome function (e.g. chloramphenicol or lincomycin) or of cytoplasmic ribosome function (e.g. cycloheximide) to investigate the contribution of the two classes of protein synthetic machinery to the chloroplast protein complement in plant tissues. Most of the published data indicate that the majority of chloroplast proteins are synthesised on cytoplasmic ribosomes (Chua & Gillham, 1977; Ellis, 1977; Barraclough & Ellis, 1979; Ellis, 1981).

Results obtained with the types of *in vivo* experiment described above have been confirmed, and in many cases extended by more direct experiments involving the synthesis of either plastid-encoded or nuclear-encoded proteins *in vitro*. Blair and Ellis (1973) demonstrated light-driven protein synthesis in intact isolated chloroplasts of *Pisum sativum*, and established that the major soluble translation product was the large subunit of RuBPCase. Two-dimensional gel analysis of the soluble polypeptides labelled with [^{35}S]-methionine during protein synthesis reveals about 80 other labelled spots (Ellis, 1977). In another type of *in vitro* protein synthesis experiment, Hartley *et al* (1975) translated spinach chloroplast RNA in a cell-free protein synthesising extract of *E. coli*. These workers found that a number of polypeptides were synthesised, including the large subunit of RuBPCase and the 32 KD polypeptide of the photosystem II complex.

The *in vitro* synthesis of nuclear-encoded chloroplast polypeptides has been investigated using cell-free translation systems derived from wheat-germ or rabbit reticulocyte lysates. All nuclear-encoded plant mRNAs studied to date are polyadenylated, whereas plastid mRNAs characteristically are not (Westhoff *et al.*, 1981; Herrmann *et al.*, 1982). Hence, the cell-free synthesis of nuclear-encoded polypeptides can be carried out by programming one of the above translation systems with polyadenylated RNA, which is readily separated from poly(A)-minus and ribosomal RNA by oligo (dT) cellulose chromatography. Immunoprecipitation of a cell-free translation product by anti-serum raised against a chloroplast polypeptide constitutes evidence that the polypeptide is nuclear-encoded. The risk of mistaken assignment of the intracellular origin of the mRNA (for example by translation of a plastid mRNA species that is polyadenylated) is small: Bottomley *et al.* (1976) have shown that spinach chloroplast RNA is readily translated by an *E. coli* cell-free extract, but not by a wheat-germ translation system. This result shows that chloroplast mRNAs, as well as ribosomes, are of the prokaryotic type and are therefore translated in prokaryotic, but not eukaryotic, cell-free translation systems.

A number of experiments carried out using protein synthesis inhibitors and *in vitro* translation systems have demonstrated that in higher plants the RuBPCase small subunit is synthesised in the cytoplasm (e.g. Barraclough & Ellis, 1979; Roy *et al.*, 1977).

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3. MECHANISMS OF PROTEIN LOCALISATION

A. Introduction

Work on a wide variety of organisms has shown that all eukaryotic cells are composed of a number of distinct, membrane-bound subcellular compartments, some of which are common to all cells whereas others are restricted to certain cell types. Protein synthesis, however, takes place only in the cytoplasm and, to a limited extent, in mitochondria and plastids. Hence, the biogenesis and maintenance of noncytoplasmic compartments such as the endoplasmic reticulum, Golgi apparatus, lysosome, glyoxysome and peroxisome is entirely dependent on the transport of proteins to these organelles from the cytoplasm. The biogenesis of mitochondria and plastids involves the activities of both cytoplasmic and organellar protein synthetic systems.

In addition, the function of many cell types involves the secretion of specific proteins from the cell; such proteins must be transported to the plasma membrane and released into the medium.

The processes by which proteins are transported to their correct location are selective in that few, if any, proteins are found in more than one cellular compartment. A number of the transport systems have received a great deal of attention in recent years, and these will be considered in more detail below.

B. Transport of secretory proteins

The intracellular pathway followed by proteins secreted from eukaryotic cells can be summarised as follows:

1. Synthesis of the polypeptide on polysomes bound to the rough endoplasmic reticulum.

2. Segregation of the polypeptide into the lumen of the rough endoplasmic reticulum.

3. Transport of the polypeptide from the endoplasmic reticulum, through the Golgi apparatus, to secretory vesicles, which are stored until secretion.

4. Release of the polypeptide to the extracellular medium by exocytosis.

(Siekevitz & Palade, 1960; Jamieson & Palade, 1967a, b; Greene *et al.*, 1963; Tanaka *et al.*, 1980).

Using a cell-free translation system, Milstein *et al.* (1972) were the first to show that secreted proteins (in this case immunoglobulin G light chain) are initially synthesised *in vitro* as larger precursors with an N-terminal extension. Blobel and Dobberstein (1975a, b) then studied the factors required for the segregation of such precursors into microsomal vesicles (as judged by resistance to added proteases). They found that if the vesicles were present from the start of translation,

the polypeptide was transported into the vesicles and processed to the mature size. However, if vesicles were added post-translationally, no segregation or processing occurred. Thus, segregation of the polypeptide was proposed to involve the co-translational transport of the nascent polypeptide through the vesicle membrane. It was considered likely that the peptide extension (the signal sequence) contained the information required for segregation of the nascent polypeptide. On the basis of these findings, Blobel and Dobberstein (1975a, b) proposed the signal hypothesis, which can be summarised as follows:

1. The nascent polypeptide of a secretory protein contains an N-terminal signal sequence of 15-30 residues.

2. During synthesis of the polypeptide, the signal peptide initiates binding of the translation complex to the rough endoplasmic reticular membrane.

3. As translation proceeds, the nascent polypeptide chain is inserted through a pore in the membrane which arises as a result of the binding of the translation complex.

4. The polypeptide is transported vectorially across the membrane into the lumen of the endoplasmic reticulum. The signal sequence is removed proteolytically, probably before synthesis of the polypeptide is complete.

5. The ribosome dissociates from the membrane.

A notable variation on this theme emerged when synthesis and segregation of ovalbumin was studied (Lingappa et al., 1978a). Although this secretory protein was shown to be synthesised and transported in a typical manner, the cell-free translation product does not contain an extension sequence that is removed by the signal peptidase (Palmiter et al., 1978). A number of reports have suggested that ovalbumin contains instead an internal signal sequence, though various parts of the molecule have been assigned this function (Lingappa et al., 1979; Meek et al., 1982; Braell & Lodish, 1982). This finding has a number of implications concerning the mechanism of transport across the endoplasmic reticular membrane; apparently cleavage of the signal is not an essential part of the segregation process. Furthermore, the results suggest that different regions of the nascent polypeptide are recognised by the microsomal membrane receptors and the signal peptidase.

Although the signal hypothesis was proposed originally to explain the transfer of secretory proteins into the endoplasmic reticulum, a number of studies indicate that integral plasma membrane proteins are segregated by the same type of mechanism. In such cases, the polypeptides then follow the same route to the plasma membrane but, unlike secretory proteins, the polypeptides are not discharged into the extracellular medium. The best-known example is the glycoprotein (G) of vesicular stomatitus virus (VSV). This protein is synthesised on membrane-bound ribosomes and is co-translationally inserted into the microsomal membrane in a manner similar to secreted proteins. The process also invovles removal of a signal peptide (Katz et al., 1977a, b; Lingappa et al., 1978b; Rothman & Lodish, 1977). However, the

segregated protein remains anchored in the microsomal membrane and is not released into the lumen of the endoplasmic reticulum. A number of studies on other membrane proteins yielded similar results (Braell & Lodish, 1981; Kehry et al., 1980).

Two of the components involved in the translocation of polypeptides across the microsomal membrane have been purified and characterised. One is an 11S ribonucleoprotein consisting of six non-identical polypeptides and one 7S RNA molecule (Walter & Blobel 1980, 1982). This complex, termed signal recognition particle (SRP), has been shown to bind to the signal sequence as it emerges from the ribosome, thereby halting translation (Walter & Blobel, 1981). The translation arrest can be released by the addition of salt-washed microsomal membranes (Meyer et al., 1982; Gilmore et al., 1982a). Two groups have purified the membrane component responsible for relieving the SRP-mediated translation block. The component is a 72 KD membrane protein termed the SRP-receptor (Gilmore et al., 1982b) or "docking protein" (Meyer et al., 1982).

After transport into the lumen of the endoplasmic reticulum (or, in the case of plasma membrane proteins, into the membrane of the endoplasmic reticulum), the proteins are transported to the plasma membrane via the other organelles of the secretory pathway. It is generally thought that transport between the organelles takes place by formation of vesicles which bud from one organelle and fuse with another (Palade, 1975).

C. Protein localisation in bacteria

The mechanisms by which bacteria transport proteins into the cytoplasmic membrane, the periplasm and the outer membrane have been the subject of a large number of publications in recent years, and only a brief description will be given here. The field has been thoroughly reviewed by Silhavy *et al.* (1983). The mechanism employed for the translocation of most of the exported proteins which have been studied is very similar to that described by the signal hypothesis for secretion of proteins in eukaryotes. Hence the exported proteins are synthesised on membrane-bound ribosomes and are translocated in a co-translational manner, during which a signal peptide is removed from the nascent chain.

D. Transport of proteins into chloroplasts

As discussed in Section I.2F, chloroplast polypeptides are synthesised in two distinct subcellular compartments. A number of polypeptides, including for example the large subunit of RuBPCase, are synthesised on chloroplast ribosomes, whereas others, including the RUBPCase small subunit, are synthesised in the cytoplasm. About 70-80% of the chloroplast polypeptides are synthesised in the cytoplasm (Chua & Gillham, 1977). These polypeptides must pass through the envelope double membrane in order to reach their correct location within the chloroplast, with the exception of those destined for one of the envelope membranes.

Work on the synthesis and transport of chloroplast proteins has concentrated mainly on two major polypeptides: the small subunit of RuBPCase (a stromal protein) and the light-harvesting chlorophyll a/b binding protein (LHCP), an integral thylakoid membrane polypeptide. Dobberstein et al. (1977) showed that the RuBPCase small subunit in Chlamydomonas is synthesised in vitro as a larger precursor with a chain extension of about 5000 daltons. These workers programmed a wheat-germ translation system with polyadenylated RNA from Chlamydomonas, and identified the carboxylase small subunit among the translation products by immunoprecipitation. Similarly, a larger precursor to the LHCP was identified in a cell-freee translation system programmed with pea polyadenylated RNA (Apel and Kloppstech, 1978).

Preliminary work on the mechanism of chloroplast protein import suggested that the signal hypothesis could not account for the transport

of RuBPCase small subunit into chloroplasts. Roy *et al.* (1977) fractionated pea leaf polysomes into soluble and membrane-bound fractions and examined the translation products when each was used to programme a cell-free translation system. The mRNA for RuBPCase small subunit was found to be associated primarily with soluble polysomes. Subsequent *in vitro* reconstitution experiments demonstrated that the import of these precursors occurs post-translationally. Highfield and Ellis (1978) showed that if pea leaf polyadenylated RNA translation products are incubated with intact isolated chloroplasts, RuBPCase small subunit precursor (P20, molecular weight 20,000) is taken up and processed to the mature size (molecular weight 14,000). The main criterion used to demonstrate import was the resistance of the mature-size, labelled small subunit to added trypsin. Further evidence of a post-translational import mechanism was provided by the observation that import proceeded in the presence of either cycloheximide or chloramphenicol.

Using a similar import assay, uptake and processing of *in vitro* synthesised LHCP precursor has been demonstrated by Grossman *et al.* (1980).

In an attempt to establish the site of processing of RuBPCase small subunit precursor, Smith and Ellis (1979) assayed various chloroplast subfractions for processing of the *in vitro*-synthesised precursor. The processing activity was found to be soluble, suggesting that processing is a stromal event. However, it should be emphasised that the fractionation procedure used did not exclude the possibility that the processing enzyme resides in the space between the two envelope

membranes.

The protein import system of higher plant chloroplasts is not species-specific. Chua and Schmidt (1978) showed that *in vitro*-synthesised RuBPCase small subunit precursors from spinach and pea are imported and processed by isolated chloroplasts from either plant species. Other (unpublished) work from this laboratory has shown that cell-free translation products from wheat and pea, including RuBPCase precursor, are imported and processed interchangeably by isolated chloroplasts from the two species. However, no import of *Chlamydomonas* polyadenylated RNA translation products into pea chloroplasts was observed by Chua and Schmidt (1978) suggesting that the import and processing machinery is not conserved throughout the plant kingdom.

A number of cytoplasmically-synthesised chloroplast polypeptides are now known to be initially made as larger precursors in cell-free translation systems. Most of the published examples are shown in Table 1, together with several which have been identified during the course of this work. The sizes of the extension sequences vary between 2000 and 15,000 daltons, and the extension sequence of a given polypeptide often varies considerably among different species of plant. Import into chloroplasts and processing to the mature size of two precursors (those of pea plastocyanin and ferredoxin-NADP oxidoreductase) has been demonstrated by Grossman *et al*. (1982).

To date, no cytoplasmically-synthesised chloroplast polypeptide has been shown to lack an extension sequence when translated in a cell-free system. However, it must be emphasised that only a small percentage of

Table 1 Cytoplasmically-synthesised precursors of chloroplast proteins

Polypeptide	Species	Extension sequence (KD)	Reference
RuBPCase Small subunit	pea	7	Cashmore (1983)
	wheat	5	Broglie et al. (1983)
	soybean	7	Berry-Lowe et al. (1983)
	Chlamydomonas	5	Dobberstein et al. (1977)
Chlorophyll a/b binding protein	pea	6	Apel and Kloppstech (1978)
Ferredoxin-NADP-oxidoreductase	pea	8	Grossman et al. (1982)
Plastocyanin	pea	15	Grossman et al. (1982)
	wheat	7	This work, Section III.2
	barley	8	This work, Section III.2
ATP-ase CF_1 subunit	spinach	4	Watanabe and Price (1982)
Rieske Fe-S protein of cytochrome b6/F complex	spinach	7	Alt et al. (1983)
Inner envelope phosphate translocator	spinach	11	Flugge and Wessell (1984)
Inner envelope protein E36	spinach	2	Flugge and Wessell (1984)
Outer envelope protein E22	spinach	10	Flugge and Wessell (1984)

imported proteins have been studied; Grossman et al. (1982) have demonstrated the import into intact chloroplasts of over 100 cell-free translation products of pea polyadenylated RNA, but most of these are unidentified. It is worth pointing out that a number of chloroplast-encoded polypeptides are also synthesised as larger precursor molecules. To date, three thylakoid proteins have been shown to be made as precursors: the herbicide-binding 32 KD protein (Grebanier et al., 1978), cytochrome F (Alt et al., 1983) and ATP-ase subunit B (Watanabe & Price, 1982). The significance of the extension sequences is unclear.

Very little information has been published concerning the mechanism of protein import into chloroplasts. The only published study on this subject has been that of Grossman et al. (1980). These workers investigated the energy-dependence of the import process, and found that import of in vitro-synthesised chloroplast polypeptides is markedly stimulated by light. The stimulatory effect of light can be replaced by added ATP, even in the presence of uncouplers, suggesting that ATP, rather than a proton gradient, is required to drive the transport process.

Published data concerning the characterisation, specificity and mechanism of the enzyme(s) involved in the processing of imported precursors are equally scarce. The only report has been that of Dobberstein et al. (1977) who described an activity present in a soluble extract from Chlamydomonas cells which was capable of processing small subunit precursor to the mature size. The subcellular location of the activity could not be determined due to the difficulty of preparing intact chloroplasts from this alga. The algal processing reaction was

inhibited by iodoacetate but not by EDTA or by serine protease inhibitors, suggesting that the enzyme responsible was a thiol protease. However, no further details of the processing activity have since been reported.

The only other significant reports on the subject of chloroplast protein transport have involved the determination of the amino acid sequences of RuBPCase small subunit precursors from several species. The entire primary sequences of small subunit precursors from pea, wheat and soybean have been deduced from sequencing of cloned DNA molecules (Cashmore, 1983; Coruzzi et al., 1983; Berry-Lowe et al., 1982). A comparison of the extension sequences of these precursors is shown in the Appendix. It is generally assumed that the extension sequence contains some (if not all) of the information specifying transport into the chloroplast and processing to the mature size. However, no experiments have been published on this area, and no information exists as to the basis for the recognition specificity of either the putative import receptors in the chloroplast envelope or the processing enzyme(s) responsible for removing the extension sequence.

E. Transport of proteins into mitochondria

The transport of proteins into mitochondria has been intensively studied in several laboratories for a number of years, and has been the subject of several recent reviews (e.g. Schatz and Butow, 1983; Hay et al., 1984). For these reasons the abundant data on this subject will not be considered in detail here; instead, the salient features of this research area will be considered.

The biogenesis of mitochondria, like that of chloroplasts, requires the activity of both nuclear and organellar genomes. A number of mitochondrial proteins are encoded by mitochondrial DNA and synthesised within the organelle. However, the coding capacity of the mitochondrial genome is limited, and, at least in yeast, Neurospora, and rat liver, only about 10% of mitochondrial proteins are encoded in the mitochondrion. The remainder are nuclear-encoded and are imported into the organelle after synthesis in the cytoplasm. Studies on a number of such polypeptides have indicated a post-translational mechanism for mitochondrial protein import, and no clear evidence of co-translational import has been published.

As with cytoplasmically-synthesised chloroplast polypeptides, the vast majority of nuclear-encoded mitochondrial polypeptides are initially synthesised as larger precursors, though a small number are synthesised with no extension sequence (a comprehensive list is given in Hay et al., 1984). The extension sequences have molecular weights of between 500 and 10,000 daltons, and in several cases have been shown to be located at the amino-terminus. Processing of the precursor to the mature size

is usually carried out by a metal-dependent matrix protease after import into the organelle; the maturation of some intermembrane space proteins is more complex and is described in Section IV.

It is apparent that in several respects the import of proteins into mitochondria resembles chloroplast protein import. However, a major difference in the import mechanisms lies in the source of energy employed by the translocation systems; whereas the import of proteins into chloroplasts is ATP-dependent (Section I.3D), import into the mitochondrial matrix and inner membrane requires an electrochemical potential across the inner membrane (Gasser et al., 1982; Schleyer et al., 1982). Import of proteins into the mitochondrial outer membrane does not require either ATP or a transmembrane potential.

Other details of mitochondrial protein import are given later in the text, usually to serve as a comparison with data which has emerged from this study concerning analogous experiments on chloroplast protein import and processing.

4. MECHANISMS OF ACTION OF PROTEOLYTIC ENZYMES

A major characteristic of chloroplast protein transport is that the imported precursor proteins are processed to the mature size during, or shortly after, translocation across the envelope (Section I.3D). The aim of the experiments described in this thesis has been to characterise the protease(s) involved, and to provide a preliminary analysis of the reaction mechanism. For this reason, a consideration of the structural and kinetic characteristics of other, well-characterised proteases is relevant.

Proteases can be divided into two main functional classes: "general" non-specific proteases whose role is to degrade proteins, and processing proteases, which exhibit a much greater level of specificity in that they are responsible for the limited cleavage of precursor polypeptides to yield the mature form.

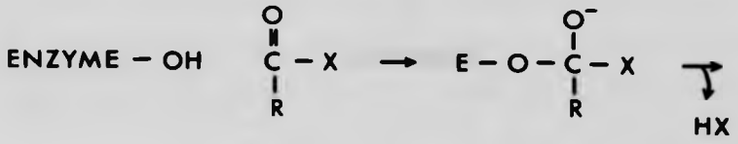
Non-specific proteases will be considered first, and several of the best-known examples, together with the substrate residues at which they carry out hydrolysis, are listed in Table 2. Endoproteases (both non-specific and processing proteases) can be classified on the basis of the functional groups involved in the catalytic process. The four main groups are described below.

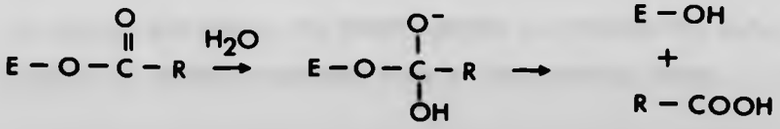
Table 2 Specificity of proteases

Protease	Molecular weight	Exo- or endo-proteolytic	Substrate Residues recognised
Trypsin	23,800	Endo	Lys or Arg
Chymotrypsin	24,900	Endo	Phe, Tyr or Trp
Carboxypeptidase A	34,600	Exo	Phe, Trp or Tyr
Carboxypeptidase B	34,600	Exo	Arg or Lys
Pepsin	34,600	Endo	Hydrophobic residues
Leucine aminopeptidase	250,000	Exo	Most residues except Pro
Papain	23,000	Endo	Described in text

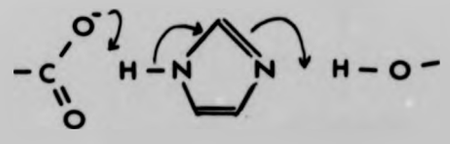
A. Serine proteases

This group are so named because they contain a catalytically-essential serine residue in the active site. Members of this class include trypsin, chymotrypsin and elastase. The reaction catalysed by serine proteases is thought to proceed via an acylenzyme intermediate, in which the catalytically-essential serine residue is acylated by the substrate as follows:





It is believed that the nucleophilicity of the serine hydroxyl group is increased by the action of a "charge relay system" which operates thus (Blow et al., 1970):



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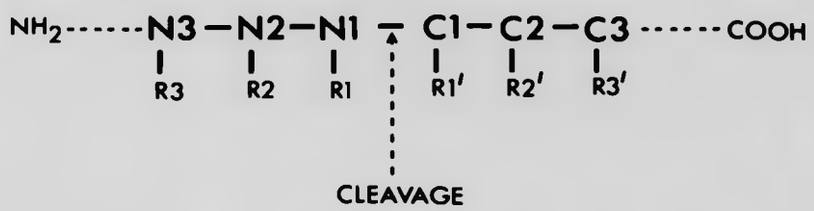
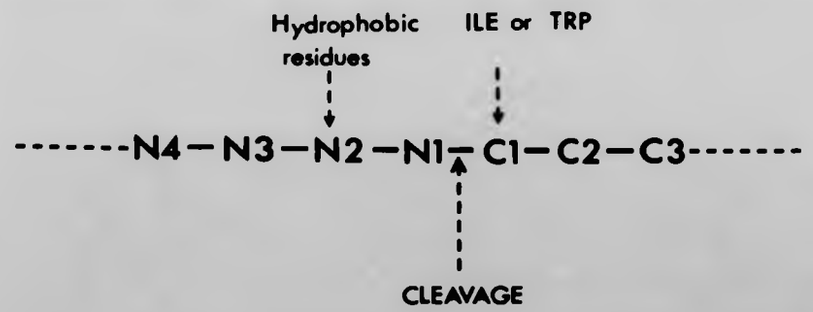
Serine proteases generally contain a binding pocket, the size and ionic characteristics of which determine the specificity of the enzyme. The binding of the peptide substrate can be represented as follows:



For serine proteases, the bound residue is usually N1, i.e. the bound residue is on the N-terminal side of the scissile bond.

B. Thiol proteases

This group of proteases differs from serine proteases by having a reactive cysteine residue in the active site. Examples include the plant enzyme papain, and the lysosomal enzymes cathepsin B1 and B2. The best studied thiol protease is papain, whose reaction mechanism has been investigated by a number of workers (Drenth et al., 1971a; Glazer and Smith, 1971; Drenth et al., 1971b; Lowe, 1976). The reaction mechanism is similar to that of serine proteases, except that an active site cysteine is acylated instead of a serine residue. However, the basis for the specificity of the enzyme is markedly different. Berger & Schechter (1970) have shown that the active site can accommodate seven amino acids, four on the aminoterminal side of the scissile bond (N1 to N4) and three on the carboxyterminal side (C1 to C3). Unlike the serine proteases that bind residue N1, papain is specific for hydrophobic residues in the N2 position. Alecio et al. (1974) have shown that there is also a specificity for isoleucine or tryptophan in the C1 site, as shown below:



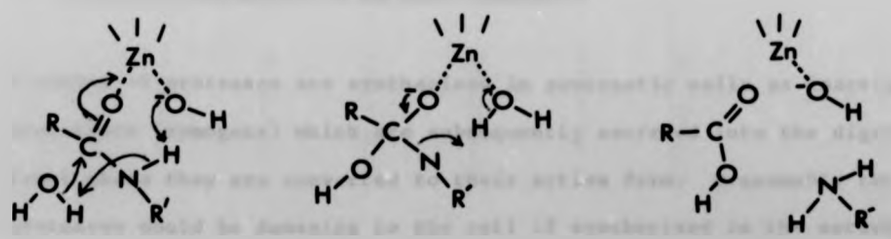
C. Acid proteases

This group of proteases derives its name from the fact that the enzymes function at low pH. Members of the group include pepsin, chymosin (rennin) and cathepsin D. The reaction mechanism of acid proteases is poorly understood, but studies on pepsin have shown that two catalytically-active aspartate residues are involved (Denburg et al., 1968; Cornish-Bowden & Knowles, 1969). These aspartate residues are thought to participate in the formation of both acylenzyme and amino-enzyme intermediates during the course of the reaction (Antonov et al., 1974; Takahashi & Hoffman, 1975; Takahashi et al., 1974).

The active site of pepsin is thought to accommodate a number of substrate residues, probably between five and seven (Sampath-Kumar & Fruton, 1974; Zinchenko et al., 1976). The enzyme has a preference for hydrophobic amino acids on each side of the scissile bond.

D. Metalloproteases

Metalloproteases contain a metal atom or atoms in the active centre which is (are) essential for catalysis. Examples of this class include thermolysin and Pseudomonas aeruginosa elastase; both of these proteases, in common with most metalloproteases, contain zinc as the essential metal. The reaction mechanism of metalloproteases is poorly understood, but kinetic and structural evidence from a number of studies has suggested a possible role of the zinc in the catalytic process. In the proposed mechanism, the free enzyme is thought to contain a tetra-coordinated zinc complex, with three ligands contributed by the enzyme and the fourth contributed by a bound hydroxide ion. The binding of a carboxyl group of the substrate generates a penta-coordinated reaction intermediate, where upon the zinc-bound hydroxide ion acts, first as a general base, and subsequently (now as a water molecule) as a proton donor, in the breakdown of the intermediate. The role of the zinc ion is two-fold: to polarise the carboxyl bond of the substrate, and to help determine the correct alignment of the attacking nucleophile (Holmes & Matthews, 1981; Chan et al., 1982). This model for catalysis is illustrated below.



E. Processing proteases

A wide variety of proteins are initially synthesised as larger precursors which require processing to the mature size in order for the protein to carry out its biological function. The proteolytic processing of such precursor molecules differs from non-specific proteolysis in two important respects.

i) The cleavage(s) usually result(s) in the formation of an active protein, rather than the formation of inactive fragments which are further degraded (an exception to this rule is illustrated in example (iv) below).

ii) The cleavage(s) is (are) specific; the precursor is processed to the mature size but no further.

The specificity of reaction is achieved in a variety of different ways, several of which are described below.

i) Conversion of zymogens to active enzymes

A number of proteases are synthesised in pancreatic cells as inactive precursors (zymogens) which are subsequently secreted into the digestive tract where they are converted to their active form. Presumably the proteases would be damaging to the cell if synthesised in the active form. Several zymogens, together with their activating agents, are listed in Table 3. The processing of some of the zymogens is

Table 3 Conversion of zymogens to active enzymes

Zymogen	Activating agent	Active enzyme		By-products
Pepsinogen	H^+ or Pepsin	Pepsin	+	Fragments
Trypsinogen	Enterokinase or Trypsin	Trypsin	+	Hexapeptide
Chymotrypsinogen A	Trypsin + Chymotrypsin	α-Chymotrypsin	+	Amino acids
Procarboxypeptidase A	Trypsin	Carboxypeptidase A	+	Fragments
Proelastase	Trypsin	Elastase	+	Fragments

autocatalytic in that the mature forms of the enzymes are responsible for the processing of their own precursors. Since these proteases are highly reactive, "general" enzymes, the question is raised: how do the proteases cleave the precursors such that the correct mature form is produced rather than inactive fragments? The answer lies in the three-dimensional structures of the proteins, which render the mature forms of the proteases extremely resistant to proteolytic attack. The conformations of the zymogens, however, allow the extra fragments to be readily removed by the activating agents.

ii) Processing of promellitin and yeast ∝-factor precursor

Melittin is a 26-residue peptide present in honey bee venom. The peptide is secreted into the venom sac as a precursor with an aminoterminal extension of 22 residues, consisting of 11 -(X-Ala/Pro)- units in a row. Kreil et al. (1980a, 1980b) have shown that the precursor is processed to the mature size by the action of a dipeptidyl aminopeptidase which sequentially removes the dipeptides X-ala or X-pro from the free aminotermial end.

Julius et al. (1983) have presented evidence that the precursor of yeast ∝-factor mating pheromone is processed to the mature size by a similar dipeptidyl aminopeptidase which sequentially removes X-ala dipeptides.

iii) Processing of viral proteins

Limited proteolysis plays an important role in the replication of many types of virus. The formation of viral proteins during the replication of single-stranded RNA viruses (picornaviruses and togaviruses) involves the processing of a giant polyprotein precursor (Korant et al., 1978). A different system operates in adenovirus (a DNA-containing virus) where Weber (1976) has shown that a number of precursor proteins are synthesised. The precursors are processed by a common viral-encoded protease during the final stages of replication. Comparison of proprotein sequences at the cleavage sites suggests that the protease recognises a consensus sequence of - gly - gly - ala -, with the cleavage occurring between glycine and alanine (Sung et al., 1983).

iv) Protein degradation during spore germination

The germination of spores of Bacillus and Clostridium species is accompanied by extensive degradation of spore protein, thereby supplying amino acids for protein synthesis (Setlow, 1975a). The major substrates for this degradation in Bacillus megaterium are three low molecular weight (7000 to 9000) proteins, termed A, B and C, which account for approximately 15% of total protein in the dormant spore (Setlow, 1975b). During the first few minutes of germination, a spore-specific endoprotease cleaves the A, B and C proteins into a few large oligopeptides which are then degraded to free amino acids by other proteases (Setlow, 1976; Postemsky et al., 1978). The protease has been isolated and shown to be highly specific; the enzyme acts on the

A, B and C proteins *in vitro* but has no activity on a variety of other peptide and protein substrates (Setlow, 1976). Analysis of the cleavage sites reveals a consensus sequence which is attacked by the proteases as follows:

CLEAVAGE
↓
- glu - X - Y - ser - glu -

where X is either Ile or Phe and Y is either Gly or Ala (Setlow *et al.*, 1980).

v) Signal peptidase

The transport of secretory proteins in eukaryotic cells and bacteria involves the removal of the "signal peptide" during the translocation of the nascent polypeptide through the endoplasmic reticular or bacterial membrane (Sections I.3B and I.3C). The signal peptidase of *E. coli* has been purified to homogeneity by Zwizinski and Wickner (1980). The enzyme has a molecular weight of 37,000, and has been shown to process the precursor forms of several other exported proteins of both prokaryotic and eukaryotic origin (Wolfe *et al.*, 1982; Zwizinski *et al.*, 1981).

The primary sequences of over fifty secreted preproteins have been determined (e.g. Hortin & Boime, 1982; Mercier *et al.*, 1978; Sugimoto *et al.*, 1977). The signal sequences vary in size from 15 to 29 residues, and show very little sequence homology that could represent a targeting site for the signal peptidase, apart from a generally high

proportion of hydrophobic residues. Furthermore, the final amino acid of the signal sequence (at which cleavage occurs) can be variable, though the side-chain involved is almost always small and uncharged (e.g. alanine, glycine, cysteine or serine). A large number of studies have been carried out using mutagenic techniques to alter the structure of the preproteins (reviewed in Silhavy *et al.*, 1983), but the mode of action of the signal peptidase remains unclear.

5. AIMS AND APPROACHES

The aim of this project was to investigate the transport and processing of cytoplasmically-synthesised chloroplast precursor polypeptides. The approach taken was to characterise the mechanism and role of the processing of the RuBPCase small subunit precursor (P20). Smith (1980) showed that this precursor is processed to the mature size by a soluble extract of pea chloroplasts. The proposed project ws to initially purify the processing activity as fully as possible, using as an assay the processing of *in vitro*-synthesised P20 to the mature size. The availability of cDNA clones encoding this polypeptide (prepared by Bedbrook *et al.*, 1980) made feasible the preparation of hybrid-selected P20 mRNA, thereby allowing the study of the processing reaction without resorting to immunoprecipitation techniques to identify the precursor and mature forms of the *in vitro*-synthesised polypeptide.

Having purified the processing activity, it was intended to characterise the processing enzyme(s) as fully as possible in terms of physical properties, specificity and reaction mechanism, a major objective of these studies being the identification of inhibitors of the processing reaction. These inhibitors could then be used to study the action of the processing enzyme *in situ* during the import of precursors by intact isolated chloroplasts.

Experiments of the kind described here were considered likely to shed light on a number of facets of chloroplast protein import, including:

i) the physical properties of the enzymic activity responsible for the processing of P20 to the mature size, and the number of enzymes involved;

ii) the precursor- and reaction-specificity of the processing enzyme(s);

iii) the species-specificity of the processing enzyme(s);

iv) the location of the processing enzyme in the intact chloroplast;

v) the role, if any, of the processing step in the transport of the precursor into the chloroplast;

vi) the mechanism of the processing reaction.

During the course of this work, a number of reports were published detailing the primary sequences of small subunit precursor from several species of higher plant (see Appendix). A comparison of the extension sequences reveals a number of conserved features which are considered likely to represent functionally significant areas of the molecule. Experiments were therefore devised to test the effects on processing and import of substituting the residues involved, in the hope that these results would give a preliminary indication of the basis for the specificity of the processing enzyme.

SECTION II - MATERIALS AND METHODS

SECTION II - MATERIALS AND METHODS

1. CHEMICALS

All materials used were of the highest analytical grade available. The source of specific reagents is given below.

BDH Chemicals Ltd., Poole, Dorset: Acrylamide, polyacrylamide, ammonium persulphate, ethidium bromide, sodium dodecyl sulphate, N-(3-nitrobenzyloxymethyl)-pyridinium chloride (NBPC), tetraethylammonium chloride.

Boehringer Corporation (London) Ltd., Lewes, Sussex: Micrococcal nuclease from Staphylococcus aureus.

Difco Laboratories Ltd., Detroit, Michigan, USA: Bactotryptone, bactoagar.

Eastman Kodak, Rochester, New York, USA: N,N'-methylene bisacrylamide, N,N,N,N'-tetramethylene diamine (TEMED).

Fisons Scientific Apparatus, Loughborough, Leicester: Formamide, dimethyl sulphoxide (DMSO).

Oxoid Ltd., London: Nutrient Broth, yeast extract.

Amersham International plc, Amersham, Buckinghamshire: L-[^{35}S]-methionine (1000 Ci/mmole).

Pharmacia (GB) Ltd., London: Percoll, Sephacryl S300 superfine, DEAE Sephacel, high molecular weight calibration proteins for gel filtration.

Sigma Chemical Co. Ltd., Poole, Dorset: Adenosine triphosphate (ATP), creatine phosphate, guanidine triphosphate (GTP) creatine phosphokinase, spermine hydrochloride, spermidine hydrochloride, L-amino acids, canavanine, azetidine-2-carboxylic acid, 1,10-phenanthroline, phenyl methyl sulphonyl fluoride (PMSF), iodoacetate, chloramphenicol, dithiothreitol, transfer RNA (E. coli K12), poly-adenylic aid (poly(A)), trypsin, soybean trypsin inhibitor, bathophenanthroline disulphonate, thialysine, coomassie brilliant blue R, ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetracetic acid (EGTA).

Uniscience Ltd., Cambridge: Oligo(dT)-cellulose.

2. GROWTH OF PLANTS

Pea seeds (*Pisum sativum* var. Feltham First) were obtained from S. Dobie & Son Ltd., Llangollen. The seeds were sown in compost (J. Arthur Bowers Compost, from Lindsay and Kestevens Ltd., Saxilby, Lincoln), watered, and placed under "warmwhite" fluorescent lights (Phillips) with a 12 h photoperiod. Light intensity was approximately 50 $\mu E/m^2/sec$ and the temperature of the growth room was maintained at $20^{o}C + 2^{o}$. Water was applied to the compost daily. The age of seedlings was measured from the time of sowing.

3. PREPARATION OF POLY(A)-CONTAINING RNA

A. Preparation of total RNA

Total leaf RNA was prepared as described by Haffner *et al.* (1978). Leaves from 10-day-old pea seedlings were removed into liquid nitrogen, recovered by filtration through one layer of muslin, and weighed. The leaves were then quickly ground using a mortar and pestle which had been acid-washed. The tissue was then transferred to a plastic beaker and for each 10 g of leaves was added 35 ml liquified phenol (80% w/v), and 35 ml 100 mM Tris-HCl, pH 7.5, 0.5% (w/v) SDS. The mixture was blended with a "Polytron" homogeniser (Northern Media Supplies, Hull) at a setting of 7 for 10 sec. The mixture was centrifuged at 1500 g for 10 min at 12°C, brake off. The upper aqueous phase was removed and the remaining phenol phase re-extracted with one volume of buffer. The pooled aqueous phases were then re-extracted with one volume of liquified phenol. Solid NaCl was added to 200 mM and 2 volumes of absolute ethanol added. Total nucleic acids were precipitated overnight at -20°C. The precipitate was collected by low-speed centrifugation and washed at least three times with 70% ethanol. The pellet was then allowed to dry in a vacuum dessicator and was dissolved in a minimum volume of sterile distilled water. RNA was precipitated by the addition of 150 mg solid NaCl per ml of RNA solution. The mixture was left overnight at 4°C. The precipitate was collected by low speed centrifugation and the pellet was washed once with 2.5 M NaCl and three times with 70% ethanol, all at 4°C. The RNA pellet was then dried, dissolved in sterile distilled water and re-extracted by salt precipitation as described above. The final pellet was dried, dissolved

in 200 mM NaCl, and precipitated with 2 volumes of ethanol. This preparation was stored at -20°C and used in the isolation of poly(A)-enriched RNA.

B. Preparation of poly(A)-enriched RNA

Poly(A)-enriched RNA was prepared by oligo(dT)-chromatography using a method based on that of Aviv and Leder (1972). Total RNA (Section II.3A), stored as an ethanol precipitate at -20°C, was collected by low-speed centrifugation, dried, and dissolved in loading buffer:

400 mM	LiCl
0.4% (w/v)	SDS
10 mM	Tris-HCl, pH 7.6

The RNA solution was then loaded onto a column containing 1 g of oligo (dT)-cellulose and the column was washed with loading buffer at room temperature. The effluent was passed through an LKB Uvicord which monitored the absorbance at 254 nm. When the absorbance of the effluent was zero, the bound RNA was collected by washing the column with elution buffer at 37°C:

0.4% (w/v)	SDS
10 mM	Tris HCl, pH 7.6

LiCl (4 M) was added to the eluate to give a final concentration of 200 mM, and the poly(A)-enriched RNA precipitated with 2 volumes of

redistilled ethanol and left at -20°C overnight. The precipitate was collected by low-speed centrifugation, washed three times with 70% redistilled ethanol and finally dissolved in a small volume of sterile distilled water. Poly(A)-enriched RNA was stored at -80°C.

4. PREPARATION OF PLASMID DNA ENCODING SMALL SUBUNIT PRECURSOR

Plasmid DNA, containing sequences encoding P20, was prepared according to Clewell (1972) from E. coli clones. The clone (pSSU160) had been prepared by Bedbrook et al. (1980), and contained near-full length cDNA prepared from P20 mRNA. The P20 DNA had been inserted into the plasmid pBR322; clones containing this plasmid are resistant to low levels of ampicillin and therefore the pSSU160 clones were grown in nutrient broth containing this antibiotic at 30 µg/ml.

A solution of "Luria broth" containing ampicillin was prepared, containing (per litre):

10 g	bactotryptone
5 g	yeast extract
5 g	NaCl
1 g	glucose
pH 7.2	with NaOH

Bactoagar (15 g per litre) was added, and the agar was melted by autoclaving at 121°C for 20 min. The molten agar was allowed to cool considerably before the ampicillin was added. After mixing, the molten agar medium was poured onto sterile petri plates under aseptic conditions. When the plates had solidified, pSSU160 clones were applied to the plates using sterile tooth picks, again under aseptic conditions. The clones were grown overnight at 37°C, and then used to inoculate a 10 ml culture of Luria broth/ampicillin. This culture was grown overnight in an orbital shaker at 37°C, and was then used to inoculate

two 750 ml cultures of Luria broth/ampicillin. These large cultures were grown by shaking at 37°C. When the absorbance (590 nm) of the cultures reached 0.8, 100 ml 4 x Luria broth, and 0.9 ml chloramphenicol (100 mg/ml in EtOH) were added to each flask. The cultures were incubated overnight at 37°C with shaking. Cells were collected by centrifugation at 5000 x g for 10 min at 4°C, and washed in 200 ml 1 mM EDTA, 10 mM Tris-HCl, pH 8.0. The pellet was resuspended in 30 ml of the EDTA/Tris buffer and re-pelleted. The cells were suspended in 8 ml 25% (w/v) sucrose, 10 mM Tris-HCl, pH 8.0, and then mixed in a freshly prepared solution containing 45 mg lysozyme in 3 ml 0.25 M Tris-HCl, pH 8.0. The suspension was incubated for 5 min on ice, then 8 ml 0.25 M EDTA-NaOH, pH 8.0 was added. After mixing, the suspension was incubated for a further 10 min on ice. The suspension was then mixed with 9.6 ml of a solution of the following composition:

2% (v/v)	Triton
10 mM	Tris-HCl, pH 8.0
10 mM	EDTA

The mixture was incubated for 20 min on ice, inverted every 5 min, and then incubated for 2 min at 42°C. The lysate was centrifuged at 30,000 x g for 60 min at 4°C. The supernatant fraction was removed and mixed with 0.5 volumes of phenol. After mixing thoroughly, 0.5 volumes of chloroform/isoamylalcohol (24:1) was added. The mixture was shaken and then centrifuged at 3000 x g for 20 min at 12°C. The upper aqueous phase was removed and mixed with 0.1 volumes of 3 M sodium acetate/HCl, pH 5.0. The DNA was then precipitated overnight at -20°C with 2.5 volumes of ethanol. DNA was pelleted by low speed centrifugation,

washed once with ethanol, and dried in a stream of nitrogen. The pelleted nucleic acid was resuspended in 14.1 ml 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 7.05 ml of this solution were added to each of two 14 ml polycarbonate centrifuge tubes. To each tube was added 6.715 g caesium chloride; the tubes were covered with parafilm and carefully inverted until the caesium chloride had dissolved. To each tube was added 425 µl ethidium bromide (10 mg/ml). The contents were then mixed and overlaid with liquid parafin. The tubes were capped, and centrifuged in an MSE 8 x 14 rotor at 40,000 rpm for 40 hours at 15°C, brake off. The tubes were then viewed with ultra-violet light from one side, and the lower band of fluorescence (supercoiled plasmid DNA) was removed from each tube using a syringe. The sample was mixed with 2/3 volume of isopropanol (water-saturated and caesium chloride-saturated) and the mixture was centrifuged at low speed. The upper layer was discarded, and the lower layer was subjected to five more extractions with isopropanol as above. This procedure removes ethidium bromide. The DNA sample was then extensively dialysed against several changes of 5 mM Tris-HCl, pH 8.0, 0.25 M EDTA at 4°C. The concentration of DNA was determined by measurement of the absorbance at 260 nm. Total DNA content was found to be 1.1 mg.

The supercoiled plasmid DNA was linearised by restriction with Eco Rl. The sample was first mixed with 1/9 volume of the following buffer:

1 M	Tris-HCl, pH 7.2
500 mM	NaCl
50 mM	$MgCl_2$
2 mM	2-mercaptoethanol

Eco RI was then added (one unit per µg of DNA) and the sample was incubated for 3 h at 37°C. Restriction was verified by agarose gel electrophoresis of samples (10 µl) of restricted and unrestricted DNA using a "minigel" apparatus (Cambridge Biotechnology Laboratries). The gel contained 0.8% (w/v) agarose. The restricted DNA was observed to migrate more slowly than the unrestricted DNA when viewed under ultraviolet light in the presence of ethidium bromide.

The sample containing the restricted plasmid DNA was mixed with 0.5 volumes phenol (H_2O-saturated) and then with 0.5 volumes chloroform/isoamylalcohol (24:1). The mixture was centrifuged at low speed, and the upper aqueous layer was removed and mixed with 0.1 volumes of 2 M ammonium acetate and 2.5 volumes ethanol. DNA was precipitated overnight at -20°C. The DNA was pelleted and washed once with ethanol, and the final pellet dissolved in 0.2 ml 25 mM sodium phosphate buffer, pH 6.0. Dimethylsulphoxide (0.8 ml) was added, and the solution was kept on ice until the activated sephacryl S-300 was ready (Section II.5).

5. PREPARATION OF PURIFIED SMALL SUBUNIT PRECURSOR mRNA BY HYBRID-RELEASE

The method involves coupling of denatured DNA to diazobenzylmethyl (DBM)-Sephacryl S-300 beads. The procedure was obtained from Dr. R. G. Herrmann (Dusseldorf) and follows that of Alwine *et al.* (1979) for DBM-cellulose. The procedure allows preparation of relatively large quantities of hybrid-released mRNA.

Sephacryl S-300 (100 g wet) was washed on a Buchner funnel with 100 ml H_2O followed by 50 ml acetone. The material was dried at 60°C to constant weight. The dried beads were wetted with a solution containing 1 g N-(3-nitrobenzyloxymethyl)-pyridinium chloride (NBPC) and 0.3 g sodium acetate in 9 ml H_2O. The pappy mass was spread out thinly over several glass beakers and dried for 30 min at 60°C. The temperature of the oven was then raised to 135°C and the material was incubated at this temperature for a further 60 min. The dry cake was homogenised carefully with a mortar and pestle (the original size of the S-300 particles must be preserved) and the powder washed on a Buchner funnel with 100 ml toluene. The NBM (nitrobenzyloxymethyl)- S-300 was dried at 60°C.

ABM (aminobenzyloxymethyl)-S-300 was prepared from NBM-S-300 by incubation for 30 min at 60°C with 50 ml of freshly prepared solution of 20% (w/v) sodium dithionite in a sealed bottle. The suspension was then transferred to a sintered-glass filter and washed with 100 ml each of H_2O, 30% (v/v) acetic acid, and H_2O. The moist ABM- S-300 was suspended in a mixture of 3 ml H_2O/10 ml 1.8 M HCl. The suspension was kept on

ice and 100 µl amounts of a freshly prepared solution of $NaNO_2$ in H_2O (10 mg/ml) added successively (4 aliquots at 10 min intervals). The slurry was transferred into an ice-cold sintered-glass filter and washed quickly with 100 ml H_2O and then with 15 ml 25 mM sodium phosphate/DMSO (dimethyl sulphoxide) (20/80, v/v). As soon as the surface of the cake became dry, 2 g of the activated S-300 were filled into a 15 ml Corex tube and mixed with 1 ml DNA solution (Section II.4). The mixture was left at room temperature overnight. The suspension was then washed at 40°C successively with H_2O and 0.4 M NaOH for about 30 min, and finally with 100 ml 10 mM Tris-HCl, pH 8.0.

The material was pre-incubated in 2.4 M tetraethylammonium chloride, pH 8.0 for 72 h at 45°C, and then washed with 100 ml 10 mM Tris-HCl, pH 8.0. This pre-incubation procedure removes non-specifically adsorbed nucleic acids. The DBM-S-300 was pre-hybridised overnight at 41°C in 3 ml of the following solution:

50% (v/v)	deionised formamide
40 mM	PIPES (piperazine-N,N'-bis(2-ethanesulphonic acid))-NaOH, pH 6.4)
0.5 M	NaCl
2 mM	EDTA
100 µg/ml	polyadenylic acid
0.2% (w/v)	SDS

Excess liquid was removed and 100 µg of poly(A)-enriched RNA added in 2 ml of the above buffer minus the poly(A). Hybridisation was carried out at 41°C for 5 h, with occasional stirring of the suspension. The

suspension was transferred into a sterile pasteur pipette containing a glass wool plug and washed with 50 ml standard saline citrate (SSC) at 41°C:

150 mM	sodium chloride
15 mM	tri-sodium citrate

The suspension was then washed with 50 ml 0.1 x SSC at 41°C and hybridised P20 mRNA was eluted with 12 ml of the following solution at 41°C:

90% (v/v)	deionised formamide
1 mM	EDTA
0.2% (w/v)	SDS
50 mM	Tris-HCl, pH 8.0

E. coli tRNA (100 µg) was added, and then solid NaCl to a final concentration of 0.2 M. RNA was precipitated by addition of 2 volumes redistilled ethanol and the suspension left overnight at -20°C. The RNA was pelleted by low-speed centrifugation and washed three times with 70% redistilled ethanol. The RNA was dried in a stream of nitrogen and dissolved in 100 µl H_2O. Each batch produced purified mRNA sufficient for approximately 50 x 20 µl translations (Section II.6D). The RNA was stored at -80°C.

The DBM-8-300 beads were washed with 10 mM Tris-HCl, pH 8.0 and stored at 4°C in 10 mM Tris-HCl, pH 8.0, 0.02% (w/v) sodium azide. The beads were re-used many times without apparent loss of ability to hybridise P20 mRNA.

6. WHEAT-GERM CELL-FREE TRANSLATION SYSTEM

A. Preparation of the wheat-germ extract

The method used was that of Roberts and Paterson (1973). All solutions and equipment were autoclaved and the entire procedure carried out at 4°C. The germ was obtained from Harrods, London.

The endosperm present in commercial wheat-germ preparations is a potential source of ribonuclease contamination; the germ can be separated from most of the endosperm by flotation on cyclohexane/carbon tetrachloride mixtures. This method is based on that of Marcus *et al.* (1974). Carbon tetrachloride (340 ml) and 140 ml cylcohexane were poured into a 500 ml beaker and mixed. Batches (20 g) of wheat-germ were poured onto the surface, mixed with the solvents and immediately the surface was scooped off with a filter-paper disc. This material was transferred to a piece of filter paper, blotted, put in a muslin bag and dried in a stream of air.

Germ (6 g) and 6 g of glass beads (80 mesh, BDH) were ground together in a mortar and pestle for about 30 sec, then 5 ml grinding buffer was added:

50 mM	HEPES-KOH, pH 7.6
100 mM	K acetate
1 mM	Mg acetate
2 mM	Ca acetate
6 mM	dithiothreitol

The mixture was worked into a paste for about 30 sec, another 10 ml grinding buffer was added, and the mixture was transferred to 2 x 15 ml Corex tubes. The tubes were centrifuged at 30,000 g for 10 min including run-up time. The supernatant solutions were removed with a pasteur pipette and the volume quickly measured.

The following additions were then made for pre-incubation (a procedure which reduces the level of endogeneous protein synthesis in the final extract). To each 1 ml of supernatant was added:

2.5 µl	1 M	Mg acetate
50 µl	20 mM	Tris-ATP/2mM GTP
40 µl	200 mM	creatine phosphate
20 µl	100 mM	dithiothreitol

Pre-incubation was at 30°C for 10 min. The mixture was then passed through a column of Sephadex G25 (coarse, 30 x 1.5 cm) which was pre-equilibrated and eluted with the following buffer:

50 mM	HEPES-KOH, pH 7.6
120 mM	K acetate
5 mM	Mg acetate
6 mM	dithiothreitol

As soon as the eluate appeared cloudy it was collected; a volume equal to that loaded on the column was taken. The wheat-germ extract was then allowed to drop through a syring needle (21 gauge) into liquid nitrogen. These spheres were stored in liquid nitrogen.

B. Nuclease treatment of the wheat-germ extract

Wheat-germ extract was treated with nuclease to further reduce the level of endogenous protein synthesis in the translation system. The extract was removed from liquid nitrogen, thawed, and to every 100 µl was added 1 µl (15 units) of nuclease from Staphylococcus aureus (Boehringer) and 2 µl 50 mM $CaCl_2$. (The nuclease is Ca^{2+}-activated). The mixture was incubated for 15 min at 20°C. The nuclease was then inactivated by addition of 2 µl 100mM EGTA- KOH (pH 8.0) for every 100 µl mixture. The extract was re-frozen in liquid nitrogen.

C. Translation of poly(A)-enriched RNA

Translation was routinely carried out in autoclaved plastic microcentrifuge tubes, and contained components at the following final concentrations:

25%	(v/v)	wheat-germ extract
100	mM	K acetate
2	mM	Mg acetate
1	mM	Tris-ATP
100	µM	GTP
10	mM	creatine phosphate
4	mM	dithiothreitol
50	µM	every protein amino acid except methionine
250	µM	spermidine
50	µM	spermine

22.5 mM		HEPES-KOH, pH 7.6
50	µg/ml	creatine phosphokinase (Sigma)
500	µCi/ml	[^{35}S]-methionine
100	µg/ml	poly(A)-enriched RNA (optimised for each preparation of RNA)

The wheat-germ extract was the last component to be added. Incubation was for 60 min at 27°C.

D. Translation of hybrid-released small subunit precuror mRNA

The translation of hybrid-released RNA was carried out under the same conditions as that of poly(A)-enriched RNA (Section II.6C) except that [^{35}S]-methionine was included at a final concentration of 2000 µCi/ml; 2 µl of hybrid-released RNA were included for every 20 µl of total translation mix.

The composition of the translation mix was altered only where specifically indicated in the text.

E. Measurement of [^{35}S]-methionine incorporated into protein

At the end of the translation period, duplicate 2 µl aliquots were transferred to strips (1 cm x 2 cm) of Whatman No.1 paper and allowed to dry. The strips were then placed in a solution of 10% (w/v) trichloroacetic acid (20 ml per strip) and heated to boiling point. The

strips were left in the hot acid for 20 min and then washed with the same volume of 10% trichloroacetic acid at room temperature, followed by two washes with 100 ml ethanol and one with ether. The strips were dried in a stream of nitrogen gas and counted in 4 ml scintillant (Beckman NA) in an LKB Minibeta scintillation counter. Efficiency of counting was about 90%.

7. ANALYSIS OF PROTEINS BY POLYACRYLAMIDE-GEL ELECTROPHORESIS

A. SDS-polyacrylamide gel electrophoresis

The gel system of Laemmli (1970) was employed, using slab gels (17 x 17 x 0.15 cm). The resolving gel contained the following components:

20% (w/v)	acrylamide
0.2% (w/v)	bisacrylamide
0.1% (w/v)	SDS
375 mM	Tris-Hcl, pH 8.8

The total volume of the resolving gel was 48 ml per slab. Immediately before the gel was poured, 200 µl 10% (w/v) ammonium persulphate and 20 µl TEMED were added. The mixture was then poured (using a syringe), overlaid with butan-1-ol, and allowed to polymerise. The top of the resolving gel was washed with water prior to addition of the stacking gel. The stacking gel contained:

4% (w/v)	acrylamide
0.1% (w/v)	bisacrylamide
1% (w/v)	SDS
125 mM	Tris-HCl, pH 6.8

Immediately before the gel was poured, 100 µl 10% (w/v) ammonium persulphate and 25 µl TEMED were added. The gel was then poured on top of the resolving gel and a slot-former inserted. After polymerisation,

the slot former was removed and the gel mounted in the electrophoresis tank. The running buffer was as follows:

25 mM	Tris-base
192 mM	glycine
0.1% (w/v)	SDS
pH 8.3	

The samples were treated as follows: one volume of sample was mixed with one volume of 2 x sample buffer of the following composition:

125 mM	Tris-HCl, pH 6.8
2% (w/v)	SDS
10% (w/v)	sucrose
5% (v/v)	2-mercaptoethanol

The samples were boiled for 2 min and then loaded into gel slots. After overnight electrophoresis (15 h at 20 mA) the gel was removed for staining (Section II.7B).

B. Polyacrylamide gel staining

Electrophoresed proteins were routinely stained by immersion for 2 h in the following solution:

0.25% (w/v)	Coomassie brilliant blue R
50% (v/v)	methanol
7% (v/v)	acetic acid

Excess stain was then removed by washing in 40% (v/v) methanol, 7% (v/v) acetic acid. If the purpose of the experiment was to analyse proteins present in column fractions, the gel was then dried and stored. If the purpose was to analyse radioactive wheat-germ translation products, it was then fluorographed (Section II.7C).

For analysis of column fractions containing low protein concentrations, the gel was subjected to silver staining. This method is at least 100 times more sensitive than Coomassie blue staining. The method follows that of Wray et al. (1981). After electrophoresis the gel was washed for at least 24 h with 3 changes of 50% (v/v) methanol (analytical grade). The following solutions were then made up immediately before staining:

A. 21 ml 0.36% (w/v) NaOH/1.4 ml ammonia solution

B. 0.8 g silver nitrate in 4 ml H_2O

Solution B was added to solution A with stirring, the volume made up to 100 ml with H_2O, and the gel immersed for 20 min. The gel was washed with water for 5 min, and then immersed in developing solution:

2.5 ml	1% citric aid
0.25 ml	37% formaldehyde
497 ml	H_2O

The citric acid and formaldehyde were mixed thoroughly before dilution to 500 ml.

Bands became visible in approximately 15 min and were fully developed within 60 min. The gel was then immersed in 50% (v/v) methanol to prevent development of a dark background.

C. Fluorography

The method follows that of Bonner and Laskey (1974). After staining with Coomassie blue (Section II.7B) the gel was washed with 2 changes of dimethylsulphoxide and immersed for 3 h in a solution containing 2,5-diphenyloxazole (22%, w/v) in dimethylsulphoxide. The gel was then soaked in circulating tap water for 30 min and dried. Spots of radioactive ink were then placed around the outside of the gel before exposure to X-ray film (Section II.14). This procedure allowed accurate superimposition of gel and developed film.

D. Counting of gel slices for [^{35}S]-radioactivity

The position of the labelled P20 and small subunit bands in the dried gel was determined by superimposing the gel and the developed film. The appropriate slices were excised, placed in 5 ml assay vials, and covered with 2.5 ml gel slice scintillant (Benbow et al., 1972). Gel slice scintillant contains (per litre):

858 ml	toluene
42 ml	liquifier
90 ml	NCS tissue solubiliser
10 ml	H_2O

Liquifier:

42 ml	toluene
4 g	2,5-diphenyloxazole (PPO)
50 mg	1,4-di-2(5-phenoxazolyl)-benzene (POPOP)

Capped vials were incubated at 37°C for 48 h, cooled to room temperature, and counted for radioactivity in an LKB Minibeta scintillation counter. Efficiency of counting was approximately 90%.

8. ISOLATION OF CHLOROPLASTS

A. Preparation of washed crude chloroplast pellets

Chloroplasts were isolated essentially as described by Blair and Ellis (1973) from leaves of 9-10 day-old pea seedlings. Leaves were homogenised with a Polytron (Northern Media Supplies, Hull) at setting 7 for 10 seconds in sucrose isolation medium (SIM):

0.35 M sucrose
2 mM EDTA
25 mM HEPES-NaOH, pH 7.6

Solid sodium isoascorbate was added to 2 mM immediately before use. Leaves (100 g) required 400 ml SIM. The leaf homogenate was filtered through 8 layers of muslin and the filtrate centrifuged at 3200 g for 1 min at 4°C. The pellets were resuspended in a small volume of ice-cold SIM using a cotton bud, pooled, and re-centrifuged. The resulting pellet constituted "washed chloroplasts".

B. Preparation of purified, intact chloroplasts

Purified intact chloroplasts were obtained from washed chloroplasts by Percoll gradient centrifugation as described by Morgenthaler *et al.* (1975). The gradient used was one of 10% to 80% (v/v) Percoll (using an MSE linear gradient former) in a 15 ml Corex tube. The solutions in the two arms of the gradient former were made up as follows

	10%	80%
Percoll	1 ml	8 ml
H_2O	7 ml	-
5 x SRM	2 ml	2 ml

1 x SRM (sorbitol resuspension medium) contains:

50 mM HEPES-KOH, pH 8.0

330 mM sorbitol

The 80% solution was pumped into the centrifuge tube while being continuously diluted with the 10% solution.

A preparation of washed chloroplasts (Section II.8A) from 20 g pea leaves was resuspended in 2 ml SRM and layered on top of the Percoll gradient. The gradient was centrifuged at 1500 g for 15 min at 4°C. The intact chloroplasts band at a position half way down the gradient; the broken chloroplasts remain near the top. The intact chloroplasts were removed using a pasteur pipette, diluted with 4 ml SRM, and centrifuged at 3200 x g for 2 min at 4°C. The chloroplast pellet was washed once more with 4 ml SRM and finally resuspended in SRM to give a chlorophyll concentration of 900 µg/ml. Chlorophyll was determined according to Arnon (1949). Chloroplasts prepared in this way were used in studies of the uptake of P20 into intact chloroplasts (Section II.11).

9. PURIFICATION OF SMALL SUBUNIT PRECURSOR PROCESSING ACTIVITY

A. Purification strategy

The purification of the processing activity involved the use of conventional procedures which have been well characterised. The activity was purified from stromal extracts of pea leaves by ammonium sulphate precipitation, gel filtration, and ion-exchange chromatography. The detailed protocol is given in Section II.9B. The assay procedure is described in Section II.10.

The ammonium sulphate fractionation procedure was chosen after quantitatively assaying a number of different fractions for processing activity.

The molecular size of the processing enzyme dictated the choice of gel filtration medium. Sephadex G-100 proved to be unsuitable because the processing activity eluted in the void volume. Sephacryl S-300 superfine was found to be ideal because the molecular size of the processing enzyme is roughly in the middle of the fractionation range of this material (Section III.3A). The column was packed and operated according to the instructions provided with the material. The choice of column size, flow rate and fraction size was the result of a number of trial runs. Two successive gel filtration steps were found to be necessary to remove the majority of ribulose bisphosphate carboxylase; this protein comprises approximately 90% of stromal protein and is therefore by far the major contaminant.

The ion-exchange medium used was DEAE-Sephacel. This medium was found to give reproducible results and was simple to use; the gel is pre-swollen, packs easily and allows high flow rates. The column size, flow rate, fraction volume and salt gradient were optimised in a number of trial runs.

The assay for the processing activity involves polyacrylamide gel electrophoresis and fluorography, and therefore the result is not known for a number of days. For this reason it was not practicable to assay each column run for processing activity in order to determine which fractions should be pooled for the next purification step. The procedure adopted was to determine which fractions contained peak processing activity in test runs of the columns, and to calculate the eluate volume difference between those fractions and the peak ribulose bisphosphate carboxylase fractions. In subsequent column runs the fractions containing processing activity would be inferred from the elution profile of absorbance at 280 nm; in all of the column runs the peak of absorbance at 280 nm represented the elution of ribulose bisphosphate carboxylase.

B. Purification protocol

All operations were carried out at 4°C. Washed chloroplasts were prepared from 600 g of pea leaves as decribed in Section II.8A. The tissue was harvested in 6 x 100 g amounts using 400 ml SIM for each 100 g. The final pellets of washed chloroplasts were lysed in a total of 50 ml 10 mM Tris-HCl, pH 7.6 and the lysates pooled. To ensure

complete lysis the suspension was repeatedly drawn into a 10 ml narrow bore pippete. The suspension was centrifuged at 30,000 g (ave) for 30 min to pellet thylakoids and envelopes, and the supernatant solution was decanted into a conical flask. Buffer (1 M Tris-HCl, pH 7.6) was added to give a final concentration of 30 mM; this concentration of buffer is necessary to prevent a drop in pH during ammonium sulphate fractionation. The final volume of the lysate was determined after removal of small aliquots for protein estimation and the assay of processing activity.

Sufficient solid ammonium sulphate to give 40% saturation (22.6 g per 100 ml) was added over a period of 30 min with constant stirring. The solution was stirred for a further 60 min and the precipitate collected by centrifugation at 10,000 g for 10 min. To the supernatant solution was added solid ammonium sulphate to give 70% saturation (a further 18.7 g per 100 ml). The mixture was stirred for 60 min and the precipitate collected as above. The supernatant solution was discarded and the pellet resuspended in 2 ml 50 mM Tris-HCl, pH 7.6. Solid sucrose was added to give a final concentration of about 5% (w/v) and half of the sample was carefully layered on top of a Sephacryl S-300 column (100 x 1.5 cm) pre-equilibrated in 30 mM Tris-HCl, pH 7.6. The column was then eluted at 20 ml/h using the same buffer. Fractions (6-7 ml) were collected using an LKB Redirac fraction collector and acid-washed fraction tubes. An aliquot of each fraction was measured for absorbance at 280 nm and a typical elution profile is shown in Fig. 5. On the following day the remaining half of the sample was chromatographed as described above.

The fractions containing processing activity are shown in Fig. 5. Routinely, the 6 fractions immediately after, but not including, the peak ribulose bisphosphate carboxylase fraction were pooled from each of the two runs. The total volumn was measured, and solid ammonium sulphate was added to 70% saturation. The suspension was stirred for 60 min and then centrifuged at 10,000 g for 10 min. The pellet was resuspended in 1 ml 50 mM Tris-HCl, pH 7.6, solid sucrose was added to a final concentration of 5% (w/v), and the sample was re-chromatographed on the Sephacryl S-300 column as described above. The absorbance at 280 nm of the fractions was measured, and a typical elution profile is shown in Fig. 6, which also shows the elution profile of the processing activity (assayed as described in Section II.10).

Fractions (x + 2) to (x + 5) (where x = ribulose bisphosphate carboxylase peak fraction) were adsorbed to a column (2.5 x 5 cm) of DEAE-Sephacel pre-equilibrated with 20 mM Tris-HCl, pH 7.6. The column was washed with 20 ml of the same buffer and then eluted with a 160 ml linear salt gradient (20 mM Tris-HCl, pH 7.6 - 300 mM NaCl, 20 mM Tris-HCl, pH 7.6). The absorbance at 280 nm of 4 ml fractions was measured and a typical elution profile is shown in Fig. 7 , together with the elution of the processing activity. Fractions (x + 4) to (x + 8) were pooled (where x = fraction of peak absorbance at 280 nm). This preparation was stored at 4^{o}C and used for a period of up to 10 days.

10. ASSAY OF THE PROCESSING ACTIVITY

Fractions from the various column runs were assayed for processing activity by incubation with wheat-germ extract containing *in vitro* synthesised P20 (Section II.6D). Incubations contained 5 µl translation products, 10 µl column fraction and 10 µl processing buffer:

220 mM	KCl
6 mM	$MgCl_2$
100 mM	HEPES-KOH, pH 8.5

The mixture was incubated for 60 min at 27°C and then mixed with one volume of 2 x sample buffer (Section II.7A) and boiled for 2 min. Samples were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography (Sections II.7A and II.7C). If processing had taken place the fluorogram showed a decrease in the label associated with P20 and a corresponding appearance of a labelled band which co-migrates with authentic mature small subunit. The fractions containing peak processing activity were routinely identified as those which produced the greatest appearance of labelled small subunit on the fluorogram. Where necessary, the elution of the processing activity was monitored quantitatively by excising the labelled bands from the dried gel and measuring the [^{35}S]-radioactivity (Section II.7D).

To determine the specific activity of a sample from one of the purification steps (in order to calculate the degree of purification) an aliquot was diluted serially in 1.5 fold steps with 20 mM Tris-HCl, pH 7.6. A number of such diluted samples were then assayed for processing

activity as described above. The labelled bands were excised and [^{35}S]-radioactivity measured; a graph was then plotted of percentage processing of P20 versus the degree of dilution. One unit of processing activity is defined as the amount required to produce small subunit containing 20% of the radioactivity initially present in the precursor.

11. UPTAKE OF SMALL SUBUNIT PRECURSOR INTO INTACT ISOLATED CHLOROPASTS

Uptake of P20 into isolated intact chloroplasts was studied using an assay procedure modified from that of Chua and Schmidt (1978). Uptake incubation mixtures contained 10 µl wheat-germ extract containing *in vitro* synthesised P20 (Section II.6D), 10 µl SRM (Section II.8B), 5 µl 5 x SRM, 20 µl 200 mM D,L-methionine, and 100 µl purified intact chloroplasts (equivalent to 90 µg chlorophyll; Section II.8B). Mixtures were incubated under illumination (100 µE m^{-2} sec^{-1}) at 25°C with gentle shaking. After the required incubation period, the mixtures were transferred onto ice and mixed with 25 µl trypsin (1 mg/ml in SRM) and 50 µl 1,10-phenanthroline (25 mM in SRM). After incubation for 30 min at 4°C the chloroplasts were diluted with 4 ml SRM and pelleted by centrifugation at 4000 x g for 2 min at 4°C. The pellets were lysed with 100 µl sterile, distilled water containing 100 µg soybean trypsin inhibitor. The lysates were centrifuged for 10 min at 4°C in an Eppendorf microcentrifuge and the stromal supernatants were removed, mixed with one volume of 2 x sample buffer, and boiled for 2 min before analysis by SDS-polyacrylamide gel electrophoresis followed by fluorography (Sections II.7A and II.7C).

12. IMMUNOPRECIPITATION PROTOCOL

Samples of wheat-germ translation mixture to be analysed were mixed with 0.1 volumes of 10% (w/v) SDS, followed by boiling for 2 min. Each sample was then mixed with an equal volume of:

1% (v/v)	NP40
20 mM	Tris-HCl, pH 7.5
2 mM	EDTA
150 mM	NaCl

Undissolved material was removed by centrifugation for 10 min in an Eppendorf microcentrifuge. The supernatant was removed and to it was added 2 µl of pre-immune serum. The mixture was incubated at room temperature for 15 min. Protein A-Sepharose beads were suspended in the above buffer to give a 10% (w/v) suspension, and 15 µl suspension was added to the sample. The mixture was incubated at room temperature for 30 min with occasional shaking, and the beads were then pelleted by centrifugation in a microcentrifuge for 30 sec. The supernatant was transferred to a fresh tube and mixed with 4 µl antiserum and 25 µl Protein A-Sepharose suspension. The sample was mixed by continuous rotation for 60 min and the beads pelleted as above. The supernatant was discarded and the beads were washed three times with 400 µl of the following solution:

0.2% (v/v)	NP40
20 mM	Tris-HCl, pH 7.5
1 mM	EDTA
150 mM	NaCl

The beads were then washed twice with the same buffer containing 500 mM NaCl. The beads were resuspended in a mixture of 50 µl H_2O and 50 µl 2 x sample buffer (Section II.7A) with boiling for 5 min. The beads were then allowed to settle, and the supernatant was analysed by SDS polyacrylamide gel electrophoresis followed by fluorography (Sections II.7A-II.7C).

13. PURIFICATION OF PLASTOCYANIN

Plastocyanin was extracted essentially as described by Plesnicar and Bendall (1970). Pea leaves (1 Kg) were homogenised for 1 min at medium speed in a Waring blender in a mixture of:

900 g	crushed ice
100 ml	1M Tris-HCl, pH 7.6
1 L	acetone (at -20°C)

The homogenate was squeezed through 4 layers of muslin and centrifuged at 6000 x g for 5 min at 0°C. The supernatant was decanted and 1.16 volumes of cold (-20°C) acetone added with stirring, and the mixture left to settle for 20 min at 4°C. The supernatant was removed by aspiration and the precipitate collected from the lower phase by centrifugation at 6000 x g for 5 min. The pellet was resuspended in 60 ml of 60 mM Tris-HCl, pH 7.6 and dialysed against 100 volumes of the same buffer overnight. The dialysed material was then centrifuged at 30,000 x g for 5 min to remove undissolved material and loaded onto a column (18 x 2 cm) of DEAE-Sephacel equilibrated in 60 mM Tris-HCl, pH 7.6. The column was washed with 100 ml of 60 mM Tris-HCl, and then eluted with a 300 ml linear gradient of 60 mM Tris-HCl, pH 7.6 - 500 mM Tris-HCl, pH 7.6, all at 4°C. Fractions containing plastocyanin were detected by the appearance of a blue colour after addition of a drop of ferricyanide solution.

The fractions containing plastocyanin were pooled and concentrated by ammonium sulphate precipitation. Solid ammonium sulphate (60.3 g per 100 ml of solution) was added with constant stirring over a period of

30 min. The mixture was left on ice for 30 min, and the precipitate collected by centrifugation at 10,000 x g for 10 min. The pellet was resuspended in 1 ml of 20 mM sodium phosphate, pH 6.9, and loaded onto a column of Sephadex G100 superfine (100 x 1.5 cm) equilibrated in the same buffer. The column was pumped at 4 ml/hr and fractions of 4 ml collected. Plastocyanin was eluted as a blue band. The plastocyanin-containing fractions were pooled and an aliquot was analysed for purity by SDS polyacrylamide gel electrophoresis. The gel showed the presence of several stained bands, and therefore the sample was further purified by ammonium sulphate fractionation. Solid ammonium sulphate was added to the sample to 70% saturation (43.6 g per 100 ml). The solid was added over a period of 30 min with constant stirring, and the mixture left on ice for 60 min. The precipitate was collected by centrifugation at 10,000 x g for 10 min, and the supernatant was removed and mixed with ammonium sulphate to give 90% saturation (a further 16.8 g per 100 ml). The mixture was left on ice for 60 min and the precipitate collected as above. The pellet was resuspended in a small volume of 20 mM Tris-HCl, pH 7.6 and dialysed against the same buffer overnight. This preparation was pure as judged by SDS polyacrylamide gel electrophoresis.

14. AUTORADIOGRAPHY AND PHOTOGRAPHY

A. Autoradiography

Dried fluorgrams containing [^{35}S]-labelled polypeptides (Section II.7) were exposed to X-Omat S (Kodak) X-ray film at -70°C. The exposure time depended on the amount of label in the gel, but was typically 7-14 days. Film was developed in Kodak DX-80, and fixed in Kodak FX-40, according to the manufacturer's instructions. The film was washed thoroughly in tap water before drying.

B. Photography

Developed X-ray films were viewed on a light-box and photographed using 35 mm Panatomix-X film (Kodak). The film was developed for 10 min with Acutol (Paterson) and fixed for 5 min with Kodafix. The negatives were then washed thoroughly with tap water before drying. Kodabrom f4 paper was used for making prints from negatives. The paper was developed using Ilford Contrast FF, and fixed with Kodafix.

SECTION III - RESULTS AND DISCUSSION

1. PARTIAL PURIFICATION OF SMALL SUBUNIT PRECURSOR PROCESSING ACTIVITY

A. Characteristics of the wheat-germ, cell-free, protein-synthesising system

Apart from the source of wheat-germ, the preparation of a cell-free extract was carried out exactly as described by Highfield (1978). The extract was stored as spheres, approximately 10-15µl in volume, under liquid nitrogen. This extract showed no sign of loss of activity after three years of storage.

The incubation conditions used for the translation of pea-leaf poly(A)-enriched RNA were as described in Section II.6C. Since different preparations of poly(A)-enriched RNA were found to perform slightly differently, the concentration of RNA in the incubation mixture was optimised for each preparation with respect to total incorporation of [^{35}S]-methionine into TCA-precipitable material. Similarly, translation of hybrid-released P20 mRNA was optimised by testing different volumes of the RNA preparation in the incubation mixture.

Figure 1 shows the dependence of the translation system on the amount of added RNA. Programming of the translation system with poly(A)-enriched RNA gives values for incorporation of [^{35}S]-methionine into protein that are similar to those obtained by Highfield (1978). When hybrid-released P20 mRNA is used as a template, the incorporation is much-reduced even at optimal levels of added P20 mRNA. The stimulation of protein synthesis above the endogenous level caused by addition of these

Table 4 Stimulation of protein synthesis in the wheat-germ system by added poly(A)-enriched RNA and hybrid-released P20 mRNA

	[^{35}S]-methionine incorporated into protein (cpm/2 µl)	
	Translation of poly(A)-enriched RNA	Translation of P20 mRNA
No added RNA (endogenous)	5,200	10,100
RNA added (zero time)	1,500	2,400
RNA added (60 min)	370,000	17,000

Incubations were set up as described in Sections II.6C and II.6D. To determine endogenous levels of protein synthesis, RNA was replaced by water. Incorporation of [^{35}S]-methionine into protein was measured as described in II.6E.

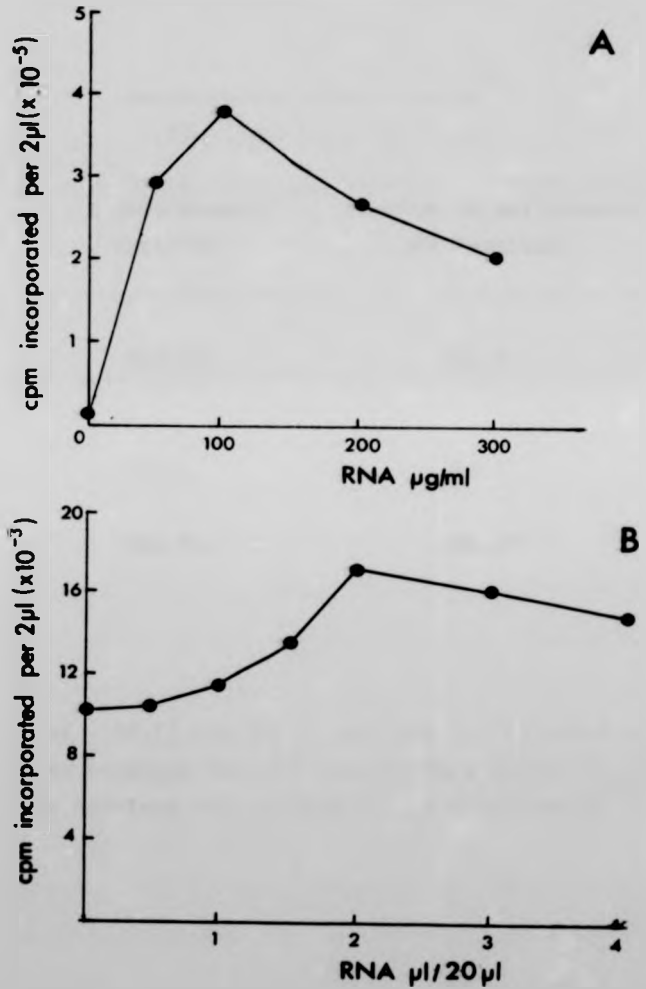

A
cpm incorporated per 2µl (x.10⁻⁵)
5
4
3
2
1
0
100
200
300
RNA µg/ml
B
cpm incorporated per 2µl (x10⁻³)
20
16
12
8
4
1
2
3
4
RNA µl/20µl

Figure 1 RNA-dependent translation in the wheat-germ incubation system

Hybrid-released P20 mRNA (Section II.5) and pea leaf poly(A)-enriched RNA (Section II.3B) were translated in wheat-germ incubation mixtures as described in Sections II.6D and II.6C respectively, except that the concentration of RNA was varied. After incubation, aliquots (2 µl) were removed for determination of [^{35}S]-methionine incorporated into protein (Section II.6E).

A: translation of poly(A)-enriched RNA.

B: translation of hybrid-released P20 mRNA.

preparations of RNA is shown in Table 4.

The translation products of poly(A)-enriched and hybrid-released RNA, as analysed by SDS polyacrylamide gel electrophoresis, are shown in Figure 2. The products translated from poly(A)-enriched RNA appear similar to those described by Highfield (1978). The major translation products, denoted as P32 and P20, have mobilities indicating apparent molecular weights of 32,000 and 20,000 respectively. Highfield and Ellis (1978) showed that P20 is the carboxylase small subunit precursor, and it is this polypeptide that is synthesised from hybrid-released P20 mRNA. The P32 polypeptide has been shown to be the precursor to the chlorophyll a/b-binding protein (A. C. Cuming, unpublished).

It can be observed in Figure 2, and in other figures shown later, that the P20 synthesised by translation of hybrid-released mRNA often migrates as a polypeptide "doublet". The reason for this is not known; translation of a given preparation of mRNA gives rise to P20 which can migrate as either a single polypeptide or a doublet in different experiments. In experiments described later in this thesis, it is shown that the intensity of both components of the doublet is decreased upon incubation with isolated processing activity, and that this leads to the appearance of mature small subunit which runs as a single band on SDS polyacrylamide gels. The same phenomenon was observed by Smith (1980). Possibly the occasional appearance of a doublet is due to some artefact of the gel electrophoresis system.

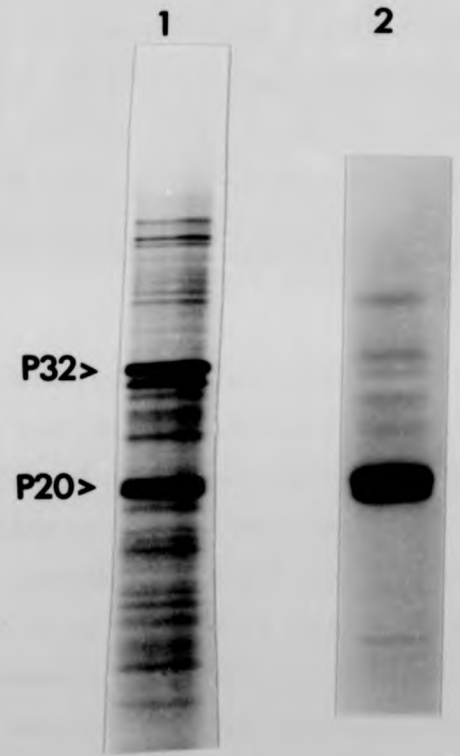

1
2
P32>
P20>

Figure 2 Translation products of poly(A)-enriched RNA and hybrid-released P20 mRNA

Hybrid-released P20 mRNA (Section II.5) and pea leaf poly(A)-enriched RNA (Section II.3C) were translated in wheat-germ incubation mixtures of total volume 20 µl as described in Sections II.6D and II.6C respectively. After incubation, the contents were mixed with one volume of 2 x sample buffer, boiled for 2 min, and analysed by SDS polyacrylamide gel electrophoresis followed by fluorography (Sections II.7A and II.7C). P20, precursor of ribulose bisphosphate carboxylase small subunit; P32, precursor of chlorophyll a/b-binding protein.

1. Poly(A)-enriched RNA translation products.
2. P20 mRNA translation products.

B. Use of in vitro-synthesised small subunit precursor to assay chloroplast extracts for processing activity

P20 processing activity was partially purified from stromal extracts of pea leaves by the procedure detailed in Section II.9B. The activity was assayed by the processing of labelled, in vitro-synthesised P20 to the mature size as judged by SDS polyacrylamide gel electrophoresis. An example of the use of this assay to monitor the elution of processing activity from one of the column steps (DEAE-sephacel chromatography) is shown in Figure 3; a number of column fractions process P20 to a form which co-migrates with authentic small subunit marker.

A number of bands are apparent in track N of Figure 3, which represents the products of wheat-germ translation mixture with no processing enzyme added. The most prominent band is P20; the other bands represent products from translation of endogenous wheat-germ mRNA, indicating that the nuclease treatment of the wheat-germ extract (Section II.6B) is not completely successful in degrading endogenous mRNA.

It can be seen in Figure 3 that processing of P20 leads not only to the appearance of mature small subunit, but also to the appearance of a less prominent polypeptide of molecular weight 18,000. (In Figure 3 this band, denoted P18, co-migrates with one of the endogenous translation products). The significance of this polypeptide is discussed in Section III.4A.

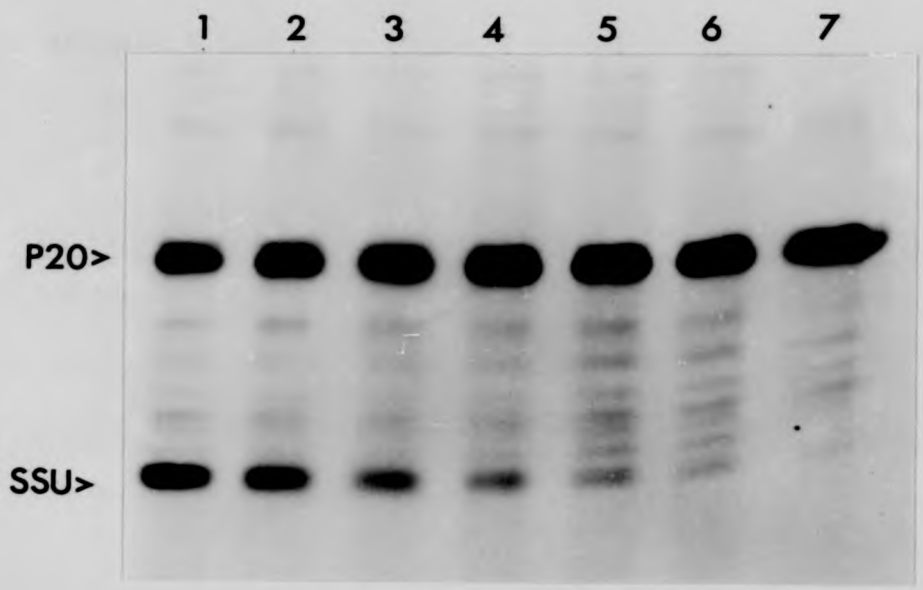
The processing reaction is dependent on the concentration of added enzyme (Fig. 4) and is specific; P20 is processed to the mature size but no further.


FR.
10
12
14
16
18
20
22
24
26
28
30
N
P20 >
P18 >
SSU>

Figure 3 DEAE-Sephacel chromatography of P20 processing activity

P20 processing activity was partially purified from a stromal extract of pea leaves by ammonium sulphate precipitation and gel filtration as described in Section II.9B (steps 1-4). The preparation was adsorbed to a column of DEAE-Sephacel and eluted with a linear salt gradient (Section II.9B, step 5). Aliquots of the eluate fractions were incubated with *in vitro*-synthesised P20 (Section II.6D) under conditions described in Section II.10. The labelled polypeptides were then analysed by SDS polyacrylamide gel electrophoresis (Section II.7A) followed by fluorography (Section II.7C). This figure shows the results of a number of the incubations; the eluate fraction number is given at the top of each track.

Symbols: P20, small subunit precursor; SSU, authentic mature small subunit; N, no processing enzyme added; P18, 18,000 mol.wt. polypeptide.


1
2
3
4
5
6
7
P20>
SSU>

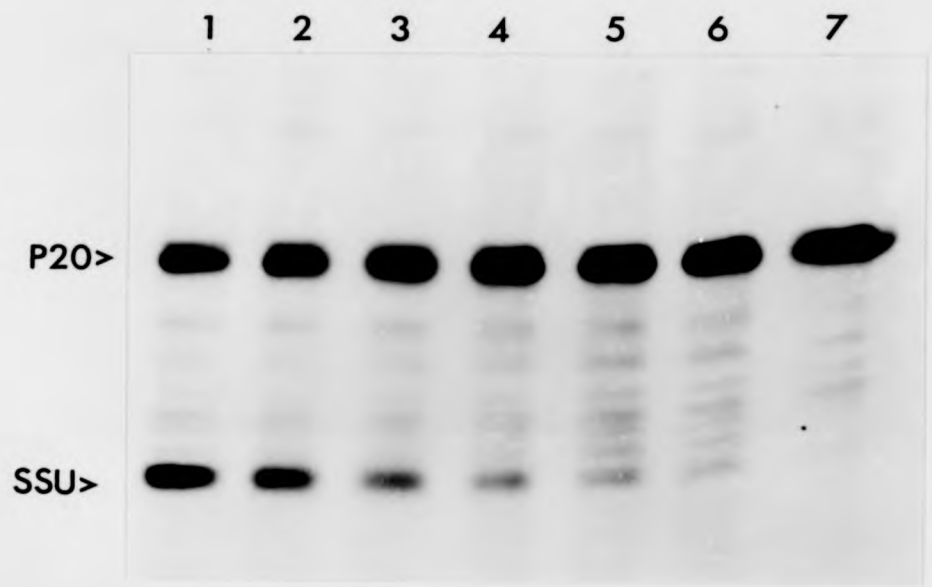

1
2
3
4
5
6
7
P20>
SSU>

Figure 4 Processing of P20 : dependence on enzyme concentration

Partially purified processing enzyme (Section II.9B) was assayed for the processing of *in vitro*-synthesised P20 (Section II.6D) as described in Section II.10. Processing enzyme was diluted with 20 mM Tris-HCl, pH 7.6 and included in the assays as follows: undiluted (track 1), diluted 2-fold (2), 4-fold(3), 8-fold (4), 16-fold (5) and 32-fold (6). Track 7 - *in vitro* synthesised P20 with no enzyme added. After incubation for 60 min at 27°C the reactions were stopped by addition of one volume of 2 x sample buffer and boiling for 2 min. Samples were analysed by SDS-polyacrylamide gel electroporesis followed by fluorography (Sections II.7A and II.7C). P20, small subunit precursor; SSU, authentic mature small subunit.

C. Partial purification of small subunit precursor processing activity from stromal extracts of pea leaves

The purification of the processing activity involved a number of chromatographic steps which are described in Section II.9B. The elution profiles of these steps, in terms of total protein (measured by absorbance at 280 nm) and of processing activity, are shown in Figures 5, 6 and 7. The purification procedures result in the removal of the majority of protein present in the initial stromal extract.

The purification of the processing activity by the column chromatography steps, as monitored by SDS polyacrylamide gel electrophoresis, is illustrated in Figures 8, 9 and 10. Samples of fractions from the two Sephacryl S-300 gel filtration steps were analysed on gels stained with coomassie blue (Figures 8 and 9). The polypeptides present in the fractions eluted from the DEAE-sephacel column (Figure 10) were analysed by silver nitrate staining of the gel (Section II.7B). This method is extremely sensitive (100 times more so than coomassie blue) and allows the detection of minute quantities of protein. The most highly purified preparations of processing activity routinely display 6-10 bands on a silver-stained SDS-polyacrylamide gel. It has not been possible to assign bands to the processing activity and therefore the molecular structure of the processing enzyme is not known.

The processing activity chromatographs as a single protein species at each step and is therefore believed to be a single enzyme. The activity behaves as a single protein on DEAE-sephacel chromatography (Figure 7) and elutes from a Sephacryl S-300 column as a single symmetrical peak

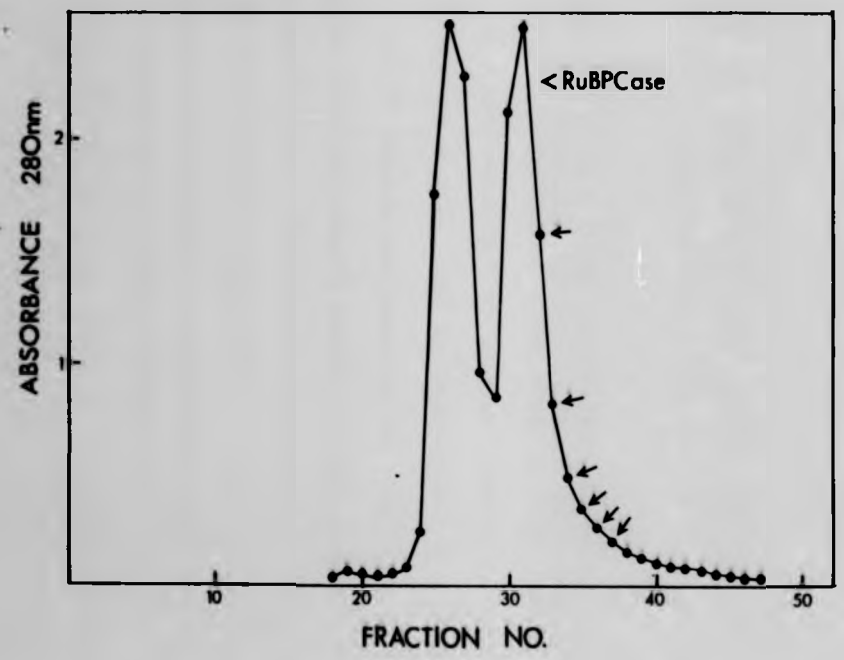

ABSORBANCE 280nm
2
1
10
20
30
40
50
FRACTION NO.
< RuBPCase

Figure 5 Fractionation of P20 processing activity by Sephacryl S-300 gel filtration (1)

P20 processing activity was partially purified from a stromal extract of pea leaves as described in Section II.9B (steps 1 and 2). The preparation was chromatographed on a Sephacryl S-300 column (Section II.9B, step 3). Fractions of 6-7 ml were collected, measured for absorbance at 280 nm, and assayed for processing of P20 as described in Section II.10. The graph shows the elution of total protein as monitored by absorbance at 280 nm. Fractions which contain processing activity are indicated by arrows; these fractions were pooled, concentrated, and re-chromatographed on the S-300 column as described in Section II.9B.

RuBPCase: ribulose bisphosphate carboxylase.

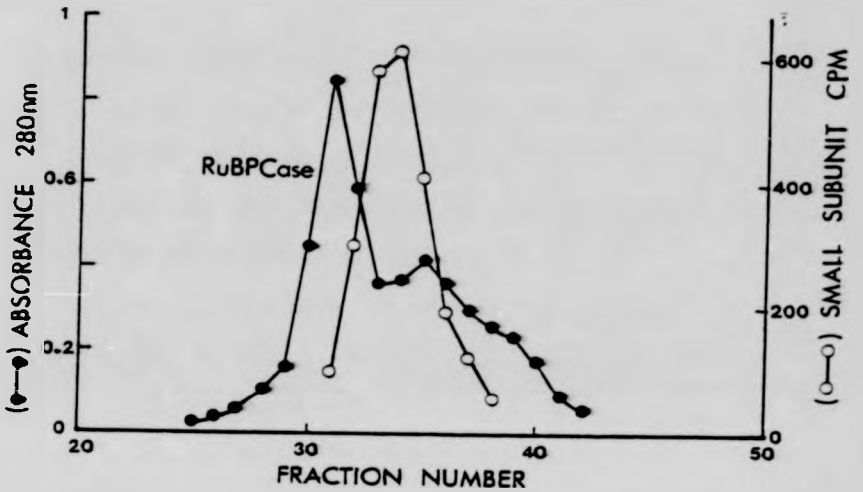

(●—●) ABSORBANCE 280nm
1
0.6
0.2
0
RuBPCase
600
400
200
0
(○—○) SMALL SUBUNIT CPM
20
30
40
50
FRACTION NUMBER

Figure 6 Fractionation of P20 processing activity by Sephacryl S-300 gel filtration (2)

P20 processing activity was partially purified from a stromal extract of pea leaves as described in Section II.9B, steps 1-3. The pooled fractions from the previous gel filtration step (Figure 4) were re-chromatographed on the Sephacryl S-300 column (Section II.9B, step 4). Fractions of 6-7 ml were collected, measured for absorbance at 280 nm, and assayed for processing of P20 as described in Section II.10. Processing was quantitated by measurement of the labelled mature small subunit produced. The figure shows the elution of total protein (as monitored by absorbance at 280 nm) and of processing activity.

RuBPCase: ribulose bisphosphate carboxylase.

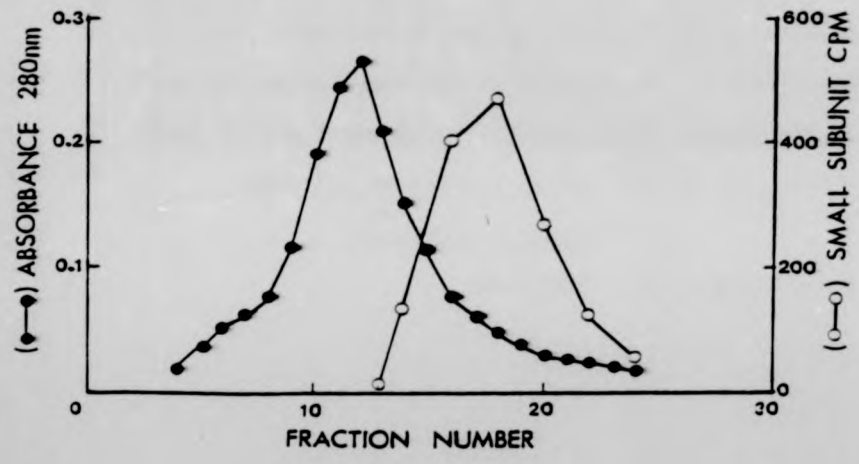
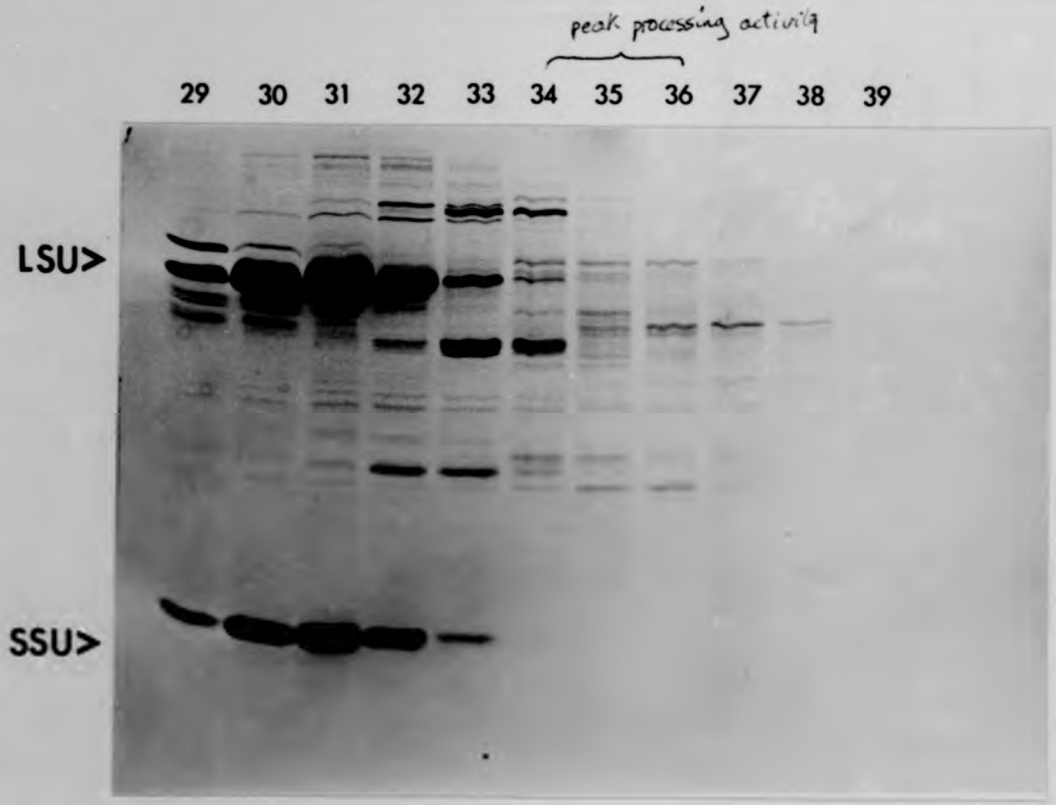

(●—●) ABSORBANCE 280nm
0.3
0.2
0.1
0
10
20
30
FRACTION NUMBER
600
400
200
0
(○—○) SMALL SUBUNIT CPM

Figure 7 Fractionation of P20 processing activity by DEAE-Sephacel chromatography

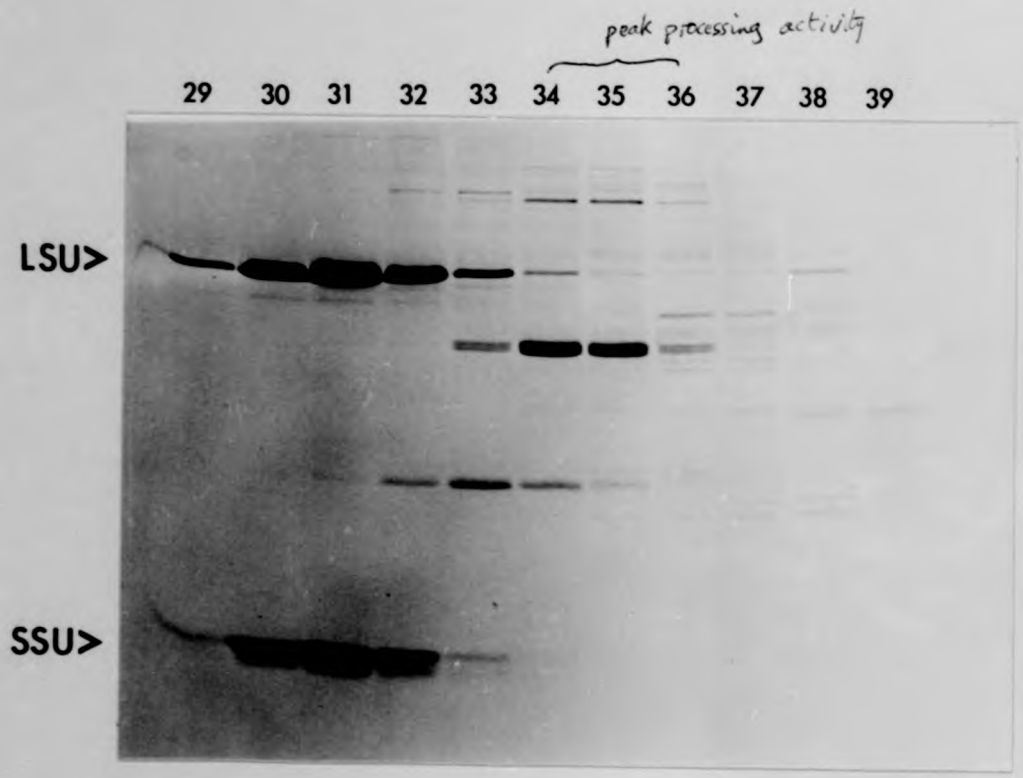
P20 processing activity was partially purified as described in Section II.9B, steps 1-4. The pooled fractions containing processing activity from step 4 were adsorbed to a column of DEAE-Sephacel and eluted with a linear salt gradient (Section II.9B, step 5). Eluate fractions were measured for absorbance at 280 nm, and assayed for processing of P20 as described in Section II.10. Processing was quantitated by measurement of the labelled mature small subunit produced. This figure shows the elution of total protein (as monitored by absorbance at 280 nm) and of processing activity.


peak processing activity
29
30
31
32
33
34
35
36
37
38
39
LSU>
SSU>


pest processing activity?
29
30
31
32
33
34
35
36
37
38
39
LSU>
SSU>

Figure 8 Fractionation of stromal proteins by Sephacryl S-300 gel filtration (1)

Aliquots (100 µl) of fractions from the Sephacryl S-300 gel filtration step described in Figure 4 were mixed with one volume of 2 x sample buffer, boiled for 2 min and analysed by SDS-polyacrylamide gel electrophoresis as described in Section II.7A. The gel was stained with coomassie blue as described in Section II.7B. Fraction numbers are given at the top of each track.


peak processing activity
29
30
31
32
33
34
35
36
37
38
39
LSU>
SSU>

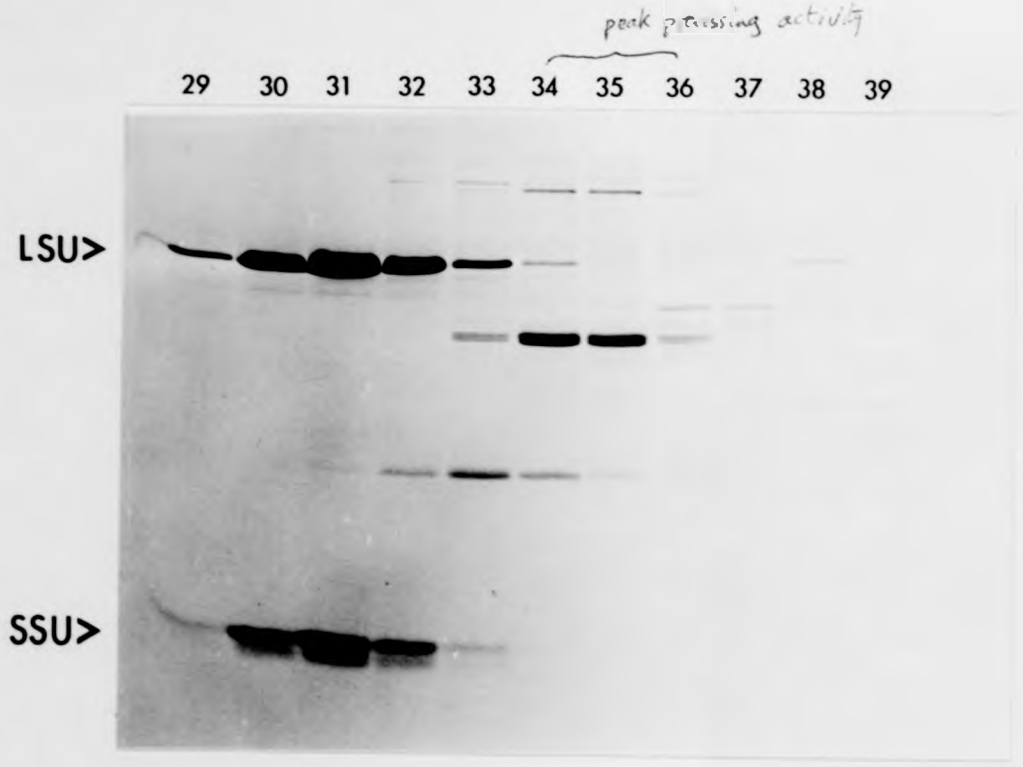

peak processing activity
29
30
31
32
33
34
35
36
37
38
39
LSU>
SSU>

Figure 9 Fractionation of stromal proteins by Sephacryl S-300 gel filtration (2)

Aliquots (100 µl) of fractions from the Sephacryl S-300 gel filtration step described in Figure 5 (Section II.9B, purification step 4) were mixed with one volume of 2 x sample buffer, boiled for 2 min and analysed by SDS-polyacrylamide gel electrophoresis (Section II.7A). The gel was stained with coomassie blue (Section II.7B). Fraction numbers are given at the top of each track.

peak processing activity

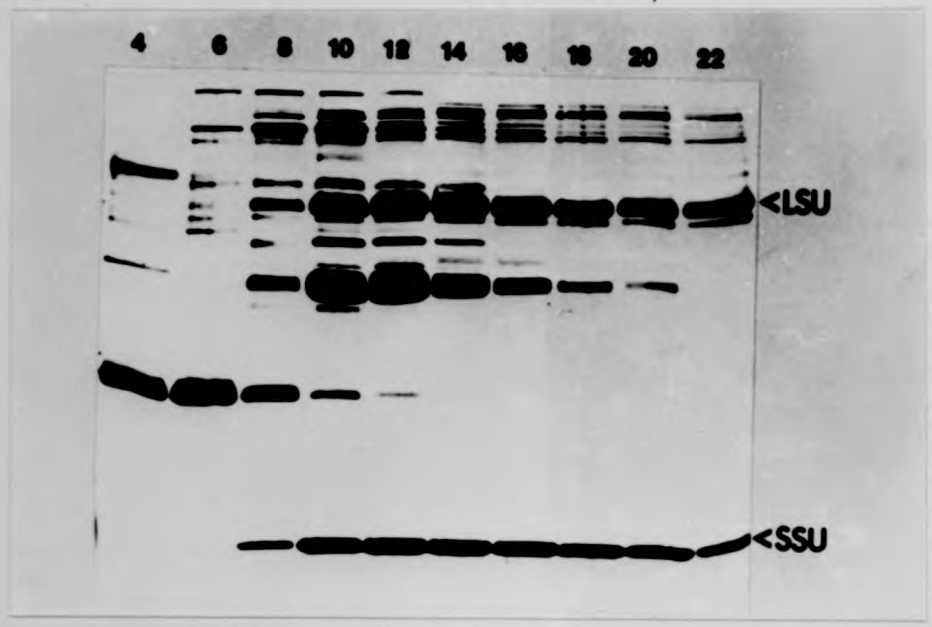


Figure 10 Fractionation of stromal proteins by DEAE-Sephacel chromatography

Aliquots (100 µl) of fractions from the DEAE-Sephacel step shown in Figure 6 (Section II.9B, purification step 5) were mixed with one volume of 2 x sample buffer, boiled for 2 min and analysed by SDS-polyacrylamide gel electrophoresis (Section II.7A). The gel was stained by the silver nitrate method (Section II.7B). Fraction numbers are given at the top of each track.

Figure 10 Fractionation of stromal proteins by DEAE-Sephacel chromatography

Aliquots (100 µl) of fractions from the DEAE-Sephacel step shown in Figure 6 (Section II.9B, purification step 5) were mixed with one volume of 2 x sample buffer, boiled for 2 min and analysed by SDS-polyacrylamide gel electrophoresis (Section II.7A). The gel was stained by the silver nitrate method (Section II.7B). Fraction numbers are given at the top of each track.

(Figure 6).

The total processing activity present in the initial stromal extract and after several of the column steps was quantitated using the procedure described in Section II.10. The results are shown in Figures 11A, B and C. The three graphs show that processing of P20 is dependent on enzyme concentration. However, an interesting feature of Figure 11A is that when an aliquot of the initial stromal extract is diluted 1.5-fold, the diluted preparation processes P20 to a greater extent than the initial extract. Further 1.5-fold dilutions show the expected relationship: the diluted aliquots show progressively less processing activity. This effect of dilution of the stromal extract has been observed in a number of experiments and may indicate the presence of an inhibitor of the processing enzyme whose effect is being diminished by the dilutions. An alternative explanation is that the high protein concentration in the initial extract is inhibitory to the processing reaction.

It must be emphasised that the quantitation of total processing activity by this assay procedure is not totally satisfactory due to limitations in the assay method. A meaningful quantitative assay of enzyme activity requires saturating concentrations of substrate and the determination of initial rates of reaction. Neither of these conditions can be satisfied in the assay of the processing enzyme because the chemical amounts of P20 synthesised by the wheat-germ system are minute and the initial rate of processing of P20 cannot be measured accurately.

A summary of the purification procedure, showing the degree of purification and the yield of processing activity at several steps, is

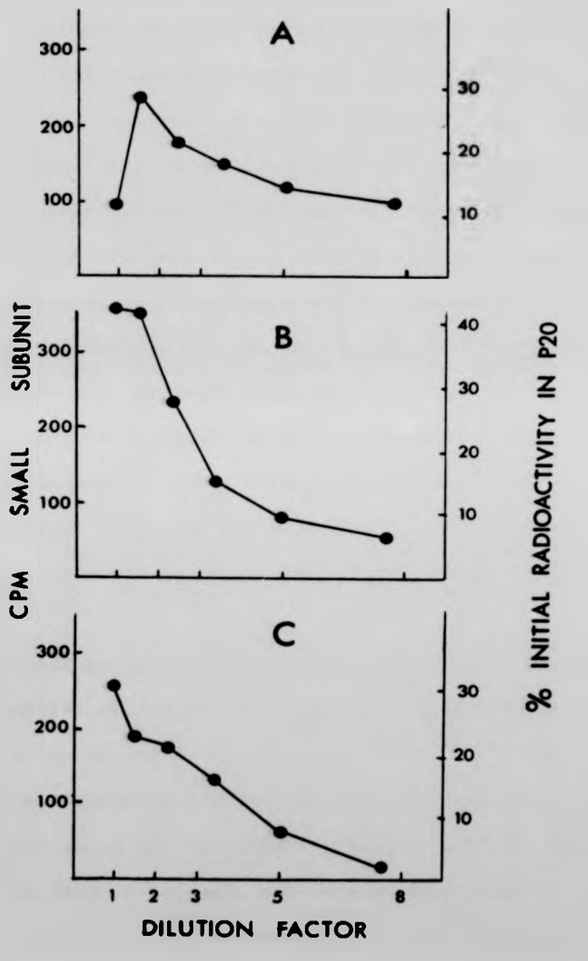

A
B
C
300
200
100
30
20
10
40
1
2
3
5
8
CPM SMALL SUBUNIT
% INITIAL RADIOACTIVITY IN P20
DILUTION FACTOR

Figure 11 Quantitation of processing activity at several stages of the purification procedure

Processing activity was partially purified from stromal extracts of pea leaves as described in Section II.9B. The initial extract, and the Sephacryl S-300 eluate (second run) and DEAE-Sephacel eluates, were quantitatively assayed for processing activity as described in Section II.10. Aliquots of each preparation were diluted, serially, 1.5-fold and incubated with *in vitro*-synthesised P20. Each reaction mixture contained 5 µl wheat-germ extract containing P20, 10 µl processing buffer and 10 µl processing activity. Each preparation of processing activity was included in the reactions diluted one-fold, 1.5-fold, 2.25-fold, 3.37-fold, 5.05-fold, and 7.07-fold. After incubation for 60 min at 27°C the incubation contents were mixed with one volume of 2 x sample buffer, boiled for 2 min, and analysed by SDS polyacrylamide gel electrophoresis followed by fluorography (Sections II.7A and II.7C). The labelled small subunit bands were excised from the dried gels and counted for radioactivity (Section II.7D). The small subunit counts were plotted against the factor of dilution, and from the graphs a dilution factor was determined at which the preparation would process P20 to produce small subunit containing 20% of the radioactivity initially in the precursor. These dilution factors were multiplied against the total volume of extract or column eluate; each ml of the preparations diluted in this way contained 100 units of processing activity (1 unit = amount which processes P20 to give small subunit containing 20% of the initial radioactivity in P20 = 10 µl under the conditions specified). Total units of activity at each of the three stages are given in Table 2.

A. Crude stromal extract.
B. Sephacryl S-300 eluate (2).
C. DEAE-Sephacel eluate.

shown in Table 5. The procedure results in a 350-fold increase in specific activity with a 33% yield. For the reasons given above these figures must be regarded as approximate.

A feature of the processing reaction that is illustrated in Figure 11 is that even the most concentrated samples of processing activity are capable of processing P20 to yield mature small subunit containing about only 40% of the radioactivity initially present in the precursor. The reason for this is that the extension sequence in P20 contains three of the six methionine residues of the precursor. Hence, processing leads to a loss of 50% of the radioactivity present in P20.

Table 5 Partial purification of small subunit precursor processing activity

Fraction	Protein mg	Total units	Specific Activity units/mg	Purification - fold	Yield %
Step 1 Crude extract	835	15,000	18.1	0	100
Step 2 $(NH_4)_2$ SO_4 precipitation	634	N.D.	N.D.	N.D.	N.D.
Step 3 Sephacryl S-300 gel filtration (1)	41.4	N.D.	N.D.	N.D.	N.D.
Step 4 Sephacryl S-300 gel filtration (2)	8.3	7,200	867	48	48
Step 5 DEAE-Sephacel chromatography	0.8	5,050	6312	348	34

N.D.: Not determined. Processing activity was purified and assayed as described in Sections II.9 and II.10. Units of enzyme activity were calculated from data shown in Fig.11. Protein determination was carried out according to Bradford (1976).

D. Discussion

The results presented in this section confirm the findings of Smith and Ellis (1979) that P20 is processed to the mature size by a soluble processing activity. This processing activity has been extensively purified and appears to consist of a single protease that is present in low molar quantities in the chloroplast. The low abundance of the protein presents problems in the purification of the enzyme, because even trace contamination from other proteins causes the appearance of a number of bands on silver-stained gels of fractions of the most highly purified preparations. For this reason it has not been possible to identify bands on SDS polyacrylamide gels which represent the processing enzyme.

The low abundance of the processing enzyme also suggests that it may prove difficult to raise antisera to the enzyme, once totally purified preparations can be obtained. Generally, milligram quantities of protein are required for raising antibodies in rabbits, whereas the yield of processing enzyme in step 5 is probably in the region of 10-100 micrograms from 600 g leaves. The availability of antisera to the processing enzyme would allow several unsolved problems to be addressed, including that of the site of synthesis of the processing enzyme. If the enzyme (or subunits of the enzyme if oligomeric) is synthesised in the cytoplasm, it would be expected that the mRNA for the processing enzyme/subunit would contain a poly(A) tail. The processing enzyme mRNA would therefore be represented in the poly(A)-enriched RNA preparation obtained by oligo(dT)-cellulose chromatography (Section II.3B). Chloroplast mRNA species do not contain poly(A) tails, and hence do not

bind to oligo (dT)-cellulose; these RNA molecules are eluted together with the ribosomal RNA molecules (Wheeler and Hartley, 1975). The *in vitro* synthesis of the processing enzyme (or subunit(s) thereof) could be studied by immunoprecipitation from the translation products of poly(A)-enriched RNA. It would be particularly interesting to know whether the enzyme is synthesised as a larger precursor; if so, what processes the processing enzyme? If the processing enzyme is not synthesised in precursor form, one can conclude that processing is not an essential step in the transport process.

A second possibility is that the processing enzyme is synthesised on chloroplast ribosomes. The *in vitro* synthesis of the enzyme could then be studied by incubating isolated intact chloroplasts in the presence of [^{35}S]-methionine under conditions such that the chloroplasts carry out protein synthesis (Ellis, 1977). The labelled processing enzyme could then be immunoprecipitated from a stromal extract of the chloroplasts.

The only published report of experiments that suggest a site of synthesis of the processing enzyme is by Feierabend and Wildner (1978). These authors showed that rye plants grown at 32^{o}C do not contain chloroplast ribosomes and therefore do not synthesise carboxylase large subunit. However, small subunit still accumulates in the plastids of these plants, suggesting that the processing enzyme must be imported from the cytoplasm.

2. SPECIFICITY OF THE SMALL SUBUNIT PRECURSOR PROCESSING ACTIVITY

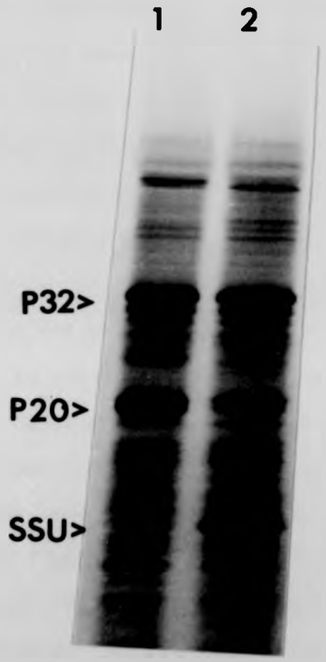
A. Processing of poly(A)-enriched RNA translation products and plastocyanin precursor

The previous section described the partial purification of a stromal protease capable of processing small subunit precursor to a form which co-migrates with authentic mature small subunit. Such an assay procedure can not exclude the possibility that the partially purified enzyme is in fact a more general protease capable of non-specifically cleaving a large number of polypeptide substrates including non-chloroplast proteins. The presence of proteases in chloroplast extracts has been demonstrated by a number of workers (for a review see Gray, 1982). Furthermore, Schmidt and Mishkind (1983) have demonstrated that if newly-imported small subunit is not assembled into ribulose bisphosphate carboxylase holoenzyme, it is rapidly degraded inside chloroplasts of Chlamydomonas reinhardii.

The partially purified enzyme is unlikely to be a general protease for several reasons. Firstly, the enzyme processes P20 to mature small subunit but no further, indicating that the reaction is specific. Secondly, during the processing reaction there is no apparent degradation of the background translation products visible on the fluorograms (due to translation of endogenous wheat-germ mRNA which "escaped" the micrococcal nuclease treatment; see Section II.6B). The processing enzyme has been further tested for non-specific proteolytic activity using as substrates, SV40 and rotavirus mRNA translation

products; again, none of these were degraded by the enzyme (data not shown). Further evidence to suggest that the isolated enzyme is a highly specific processing enzyme is presented later in this thesis. It has been found that proline and arginine residues can be substituted by amino acid analogues during the translation of P20 mRNA in the wheat-germ incubation. These abnormal precursors are both very poor substrates for the processing enzyme (see Section III.4B), suggesting that the enzyme recognises more than a single amino acid side-chain of the precursor. The processing enzyme is therefore unlikely to be a general protease such as those described in Section 14.A-D.

If the isolated processing enzyme is responsible for the processing of a number of precursors imported into chloroplats from the cytoplasm, then the enzyme would be expected to cleave a number of poly(A)-enriched RNA translation products in addition to P20. To test this possibility, the purified processing enzyme was incubated with the translation products from pea leaf poly(A)-enriched RNA (Figure 12). It can be seen that a number of bands disappear, and several new bands appear, the most prominent cleavage being that of P20 to yield mature small subunit. The other cleavages may be of other (unidentified) cytoplasmically synthesised precursors destined for the chloroplast, suggesting that the processing enzyme is responsible for the cleavage of a number of imported precursors. A more satisfactory test, however, is to assay the processing activity for the cleavage of a polypeptide which is known to be a chloroplast protein precursor. This can be done in two ways; by hybrid-release of a species of mRNA known to code for a precursor to a chloroplast protein, followed by incubation of the processing enzyme with the <u>in vitro</u> translation product of the mRNA (as with the assay of


1
2
P32>
P20>
SSU>

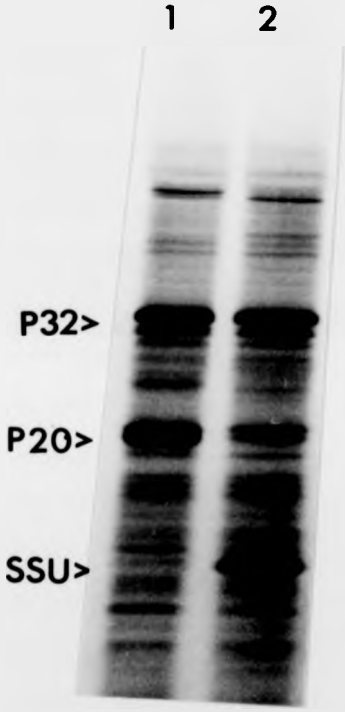

1
2
P32>
P20>
SSU>

Figure 12 Processing of poly(A)-enriched RNA translation products by partially purified processing activity

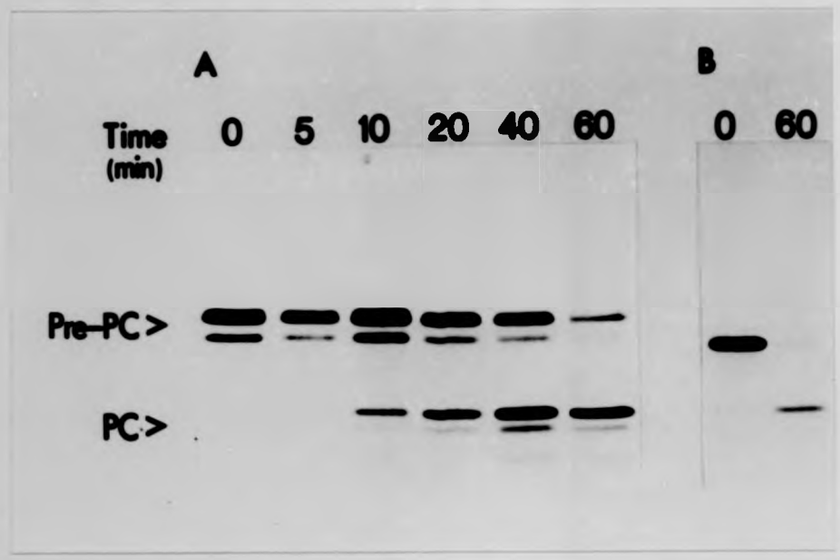
Pea leaf poly(A)-enriched RNA (Section II.3B) was translated in a wheat-germ incubation mixture (Section II.6C) of total volume 20 µl. After incubation, an aliquot (10 µl) was mixed with 20 µl processing buffer (Section II.10) and 20 µl partially purified processing activity (Section II.9B). The reaction mixture was incubated for 60 min at 27°C and then mixed with 2 x sample buffer and boiled for 2 min. The remainder of the wheat-germ incubation mixture (10 µl) was mixed with 20 µl processing bufer and 20 µl 20 mM Tris-HCl, pH 7.6, and incubated for 60 min at 27°C. The reaction mixture was then mixed with one volume of 2 x sample buffer and boiled for 2 min. Samples were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography (Sections II.7A and II.7C).

SSU: mature small subunit

1. Buffer - 60 min.
2. Processing reaction - 60 min.

processing of the small subunit precursor). A second method is to specifically immunoprecipitate a polypeptide from total poly(A)-enriched RNA translation products; processing can be demonstrated by comparing the electrophoretic mobility of the cell-free translation product before and after incubation with the processing enzyme.

Unfortunately, an investigation of the reaction specificity of the processing is hampered by the lack of suitable hybrid-released mRNA or antisera required to identify individual precursors of chloroplast proteins. The only suitable antiserum available was raised against wheat plastocyanin, a thylakoid protein encoded in the nucleus and synthesised as a larger precursor in pea (Grossman *et al.*, 1982) and wheat (Dr. J. Gray, personal communication). Partially purified processing enzyme was incubated with the translation products from wheat and barley poly(A)-enriched RNA, and processing of pre-plastocyanin to the mature size was monitored by immunoprecipitation of the precursor and/or mature forms. Figure 13 shows that the precursor from both species is processed to the mature form which co-migrates with purified plastocyanin, showing that the processing enzyme is neither precursor- nor species-specific. Processing of pea plastocyanin could not be demonstrated because the wheat antiserum did not cross-react with pea plastocyanin.


A
B
Time (min)
0
5
10
20
40
60
0
60
Pre-PC>
PC>


A
B
Time
(min)
0
5
10
20
40
60
0
60
Pre-PC>
PC>

Figure 13 Processing of wheat and barley plastocyanin precursor by partially purified processing activity

Wheat-germ incubation mixtures were set up (Section II.6C), programmed with poly(A)-enriched RNA (Section II.3B) from wheat seedlings (total incubation volume 120 µl) or barley seedlings (total incubation volume 40 µl). After incubation, each translation mixture was mixed with two volumes of processing buffer (Section II.10) and two volumes of partially purified processing activity (Section II.9B). Immediately after addition of procesing activity, samples (100 µl) were removed from each reaction mixture, mixed with 20 µl of 10% (w/v) SDS and boiled for 2 min (0 min samples). The remainder of the reaction mixtures were incubated at 27°C. After incubation for 60 min the reaction mixture containing barley RNA translation products was denatured as described for the zero time sample. Samples (100 µl) were taken at 5, 10, 20, 40 and 60 min from the reaction mixture containing wheat RNA translation products, and denatured as described for the zero time sample. Samples were subjected to immunoprecipitation with antiserum raised against wheat plastocyanin as described in Section II.12. The immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography (Section II.7A and II.7C). Pre-PC: *in vitro* synthesised plastocyanin precursor; PC: authentic plastocyanin, purified as described in Section II.13.

A. Immunoprecipitates from the reaction mixture containing wheat RNA translation products. Sample times are given above the tracks.

B. Immunoprecipitates from the reaction mixture containing barley RNA translation products. 0 min and 60 min samples are indicated above the tracks.

B. Discussion

The data presented in this section indicate that the partially purified enzyme is a highly specific protease capable of cleaving at least two chloroplast precursor polypeptides to yield the mature products. The absence of proteolytic activity against any other proteins tested strongly suggests that the observed cleavage reactions are not artefactual, and that the purified enzyme carries out the processing reactions *in vivo* as well as *in vitro*. This conclusion could be either further substantiated or brought into question by aminoterminal radio sequencing of the labelled small subunit produced in the processing reaction; the sequence should correspond exactly with that determined by Takruri *et al*. (1981) for authentic mature small subunit.

Though the processing enzyme is not precursor-specific, the data do not indicate whether all precursors imported into the chloroplast are processed by a single protease. The isolated processing enzyme would have to be assayed for the processing of as large a number of precursors as possible in order to gain a clearer understanding of the reaction specificity.

The processing of wheat and barley pre-plastocyanin by the pea processing enzyme indicates that the enzyme is not species-specific. Chua and Schmidt (1978) have shown that small subunit precursors from pea and spinach are imported and processed interchangeably by isolated intact chloroplasts, but that *Chlamydomonas* small subunit precursor is not imported by chloroplasts from higher plants. An interesting line of research would be to test the pea processing enzyme with precursors from

a number of different species and phyla in order to determine how far the processing enzyme-precursor compatibility extends.

An observation of especial interest is that the processing enzyme is capable of cleaving precursors of both stromal (e.g. carboxylase small subunit) and intra-thylakoidal (e.g. plastocyanin) proteins to the mature size. Smith and Ellis (1979) suggested that processing of P20 to mature small subunit was a stromal event, on the basis that stromal extracts contained P20 processing activity whereas washed envelopes or thylakoids did not. These results suggest that processing of pre-plastocyanin to the mature form may also take place in the stroma; if so, the mature size plastocynanin must then cross a further bilayer (the thylakoid membrane) in order to reach its correct location. A second possibility is that the precursor form is transported directly to the thylakoid compartment (perhaps by a temporary fusing of the thylakoid and inner envelope membranes) and then processed. This mechanism would require the processing enzyme to be present in the thylakoid compartment as well as the stroma, and is therefore considered improbable.

The processing enzyme has been shown to cleave a number of poly(A)-enriched RNA translation products, including P20 (Figure 12). However, as seen in Figure 12 and in a number of repeat experiments, no processing of P32 (the precursor of the chlorophyll _a_/_b_-binding protein) has been observed. The same result was obtained by Smith (1980), who could not demonstrate processing of P32 by either isolated intact chloroplasts or stromal extracts. However, several workers in this laboratory (R. Williams, A. C. Cuming and J. Bennett, unpublished results) have demonstrated the transport, processing and assembly of

this protein by isolated intact chloroplasts.

Similarly, import and processing of P32 has been demonstrated in this work (Section III.4, Figs. 27 and 29). This may indicate that a different enzyme carries out the processing of P32. Alternatively, the *in vitro* synthesised P32 may be a poor substrate for the processing enzyme in the processing assay employed. For example, the precursor may have to bind to the thylakoid membrane in order to take up the correct conformation such that processing can take place. Evidence to support the latter possibility comes from the finding that no P32 processing activity is present in either crude stromal extracts or preparations of washed thylakoids or envelopes (Smith, 1980).

3. CHARACTERISATION OF THE SMALL SUBUNIT PRECURSOR PROCESSING ENZYME

A. Molecular weight of the processing enzyme

Two methods are currently available to determine the molecular weight of a protein with a high degree of precision. In the first method, the molecular weight of the subunit(s) is determined by SDS-polyacrylamide gel electrophoresis or by sequencing methods. If the protein is oligomeric, the number and stoichiometry of the subunits is determined by one or more of a variety of available approaches (for a review see Weber et al., 1972).

In the second method, the molecular weight of purified, native protein is determined by sedimentation velocity centrifugation. The ultracentrifuge subjects a small volume of protein solution to a carefully controlled centrifugal force and records the movement of the macromolecules in the centrifugal field. From the rate of movement it is possible to calculate the sedimentation coefficient (S) and then to derive the molecular weight.

Both methods require the protein under study to be purified to homogeneity, and therefore neither is applicable to the processing enzyme. It is, however, possible to obtain an estimate of the molecular weight using gel filtration chromatography. In this procedure, molecular weight determination is carried out by comparing some elution volume parameter, such as Kav of the protein of interest, with the values obtained for several known calibration standards:

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$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where Ve = elution volume for the protein

Vo = column void volume

Vt = total bed volume

In practice it is found that for globular proteins whose size falls within the fractionation range of a gel filtration medium, a linear relationship exists between their elution volume parameters (such as Kav) and the logarithm of their molecular weights. A calibration curve is prepared by measuring the elution volumes of several calibration proteins, calculating their Kav values, and plotting Kav versus log molecular weight. The elution volume of the protein of interest is measured, its Kav calculated, and its molecular weight calculated from the calibration curve.

The calibration proteins used were:

Ferritin (horse spleen),	molecular weight	440,000
Catalase (bovine liver),	" "	232,000
Aldolase (rabbit muscle),	" "	158,000
Albumin (bovine serum),	" "	67,000

The elution volumes for these proteins were measured using the Sephacryl S-300 column described in Section II.9B. The peak fraction of each protein was found by monitoring the absorbance at 280 nm of the eluate. To ensure resolution of the peaks, the calibration proteins were run in

two separate groups, ferritin and aldolase in one run; catalase and serum albumin in the other. The elution volume of the processing activity was determined as previously described in Figure 6. The various elution parameters are shown in Table 6.

A plot of Kav versus log.molecular weight is shown in Figure 14. The molecular weight of the processing enzyme is found to be 182,000. This value must, however, be regarded as approximate; for such a determination to be accurate it must be shown that the processing enzyme has the same relationship between molecular weight and molecular size as that of the calibration standards. The fractionation of a protein by gel filtration chromatography depends on the size and shape of the protein, and not strictly on molecular weight.

Table 6 Sephacryl S-300 chromatography elution parameters

Column void volume (Vo)	165 ml
Total column bed volume (Vt)	472 ml

Elution volume (Ve) values and calculated Kav

	Ve (ml)	Kav
Ferritin	188	0.093
Catalase	209	0.163
Aldolase	223	0.21
Serum albumin	240	0.266
Processing enzyme	215	0.182

Further details are given in the text and in Fig. 14.

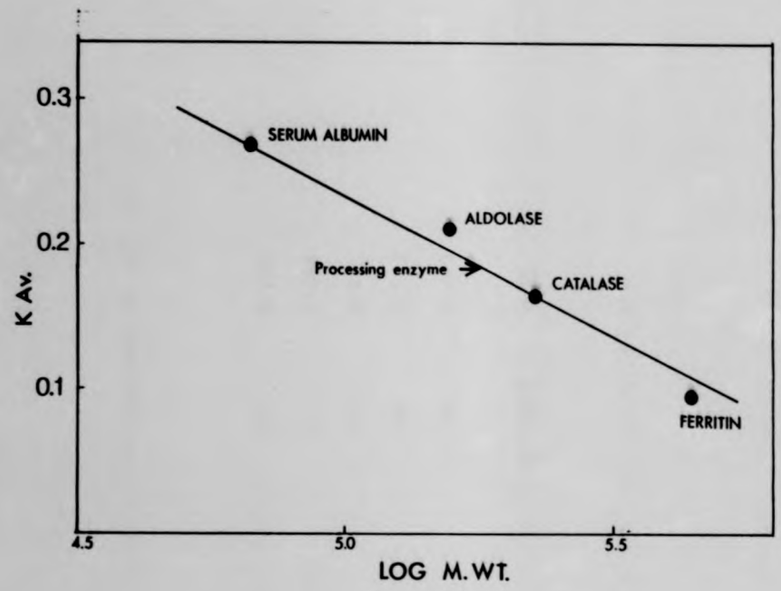

0.3
0.2
0.1
K Av.
SERUM ALBUMIN
ALDOLASE
Processing enzyme
CATALASE
FERRITIN
4.5
5.0
5.5
LOG M.WT.

Figure 14. Determination of the molecular weight of the processing enzyme by Sephacryl S-300 gel filtration

The figure shows a plot of Kav versus log.molecular weight for a number of calibration proteins. Kav for each protein was calculated from the elution volume of the protein from a Sephacryl S-300 column as described in the text. Elution volume, and hence Kav of the processing activity was determined as described in Figure 6. From the calibration curve, the molecular weight of the processing enzyme is 182,000.

B. The effect of pH on the rate of processing of P20 by the isolated processing enzyme

The pH-dependence of the processing reaction was investigated by carrying out the reaction in buffers of pH 6.5 to 10.0. Since no commonly used buffer system is capable of buffering effectively over this pH range, it was necessary to use two buffers whose effective pH ranges overlap. HEPES-KOH was used to buffer incubations at pH 6.5, 7.0, 7.5, 8.0 and 8.5. Glycine-NaOH was used to buffer incubations at pH 8.5, 9.0, 9.5 and 10.0. Processing reactions were carried out in both buffers at pH 8.5 to test the possibility that one buffer system inhibits the processing reaction relative to the other.

The buffering capacity present in the wheat-germ translation mix (Section II.6C) is provided by 22.5 mM HEPES-KOH, pH 7.6. This buffer component is diluted by a factor of five in the processing assay by the addition of two volumes of processing buffer and two volumes of processing activity (Section II.10). The final concentration of the HEPES or glycine buffers indicated above in each processing reaction was 240 mM; this was deemed sufficient to overcome the buffering capacity of the wheat-germ mixture.

The results of this experiment are shown in Figure 15. The enzyme activity increases from a low level at pH 6.5 to a peak near 9.0, and falls away sharply thereafter. The enzyme is virtually inactive at pH 10.0.

The reaction rates at pH 8.5 in the presence of the two buffer systems

A

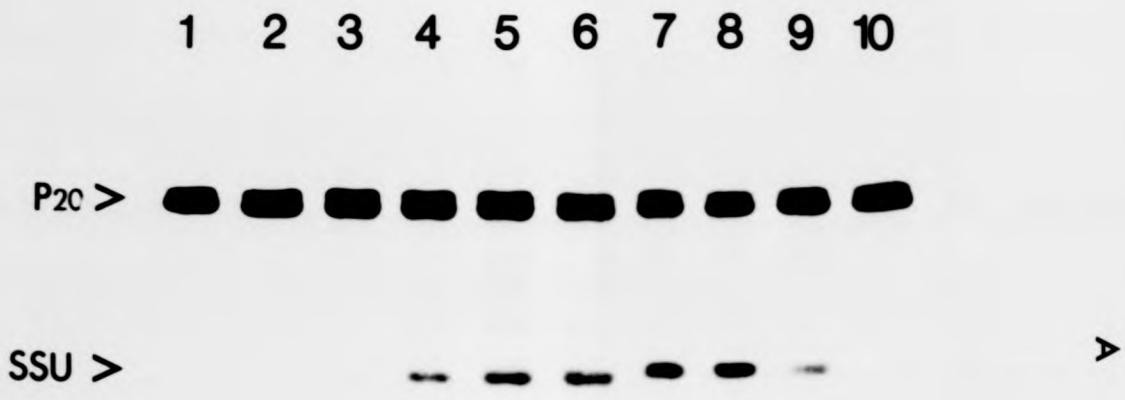

1 2 3 4 5 6 7 8 9 10
P2C >
SSU >
A

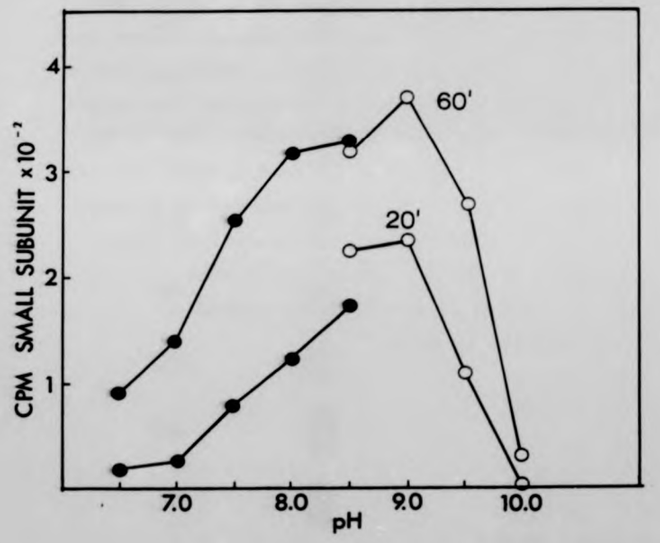
Figure 15 The effect of pH on processing of P20

Processing activity was partially purified as described in Section II.9B. Aliquots (100 µl) were dialysed at 4°C overnight against 1000 volumes of the following buffers: 300 mM HEPES-KOH, pH 6.5, 7.0, 7.5, 8.0 and 8.5, and 300 mM glycine-NaOH, pH 8.5, 9.0, 9.5 and 10.0. Nine processing incubations were set up as described in Section II.10; each incubation contained 10 µl wheat-germ extract containing *in vitro* synthesised P20, 20 µl of one of the above preparations of processing activity, and 20 µl processing buffer in which the buffer component was replaced with the same buffer, at the same pH, as that of the preparation of processing enzyme. Incubation was at 27°C. After 20 min, samples (25 µl) were removed from each reaction mixture, mixed with one volume of 2 x sample buffer, and boiled for 2 min. The remainder of each reaction mixture was incubated for a further 40 min and then mixed with 2 x sample buffer and boiled as above. Samples were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography (Section II.7A and II.7C), together with aliquots (10 µl) of wheat-germ extract containing P20 which had not been incubated with processing activity. Figure 16A shows a fluorogram of the 60 min samples from each incubation. SSU: mobility of authentic mature small subunit.

1. No processing activity added.
2. HEPES-KOH, pH 6.5.
3. HEPES-KOH, pH 7.0
4. HEPES-KOH, pH 7.5
5. HEPES-KOH, pH 8.0
6. HEPES-KOH, pH 8.5
7. Glycine-NaOH, pH 8.5
8. Glycine-NaOH, pH 9.0
9. Glycine-NaoH, pH 9.5
10. Glycine-NaOH, pH 10.0

Processing of P20 in 20 min and 60 min samples from each reaction was quantitated by excision of the labelled small subunit bands from the dried gels and counting for [^{35}S] radioactivity. Figure 16B shows a plot of extent of processing versus incubation pH from reactions carried out in the presence of HEPES-KOH (●-----●) and glycine-NaOH (o-----o).

B



are similar, indicating that one buffer was not significantly inhibiting the processing reaction relative to the other.

It should be noted that the low rates of processing at the extremes of the pH range tested (pH 6.5 and pH 10.0) are due to low rates of enzyme activity and are not caused by inactivation of the processing enzyme. Control tests have shown that the processing enzyme is not affected by dialysis overnight against buffers at pH 6.0-10.0; such preparations show no loss of processing activity when the pH is subsequently adjusted to pH 8.5 (data not shown).

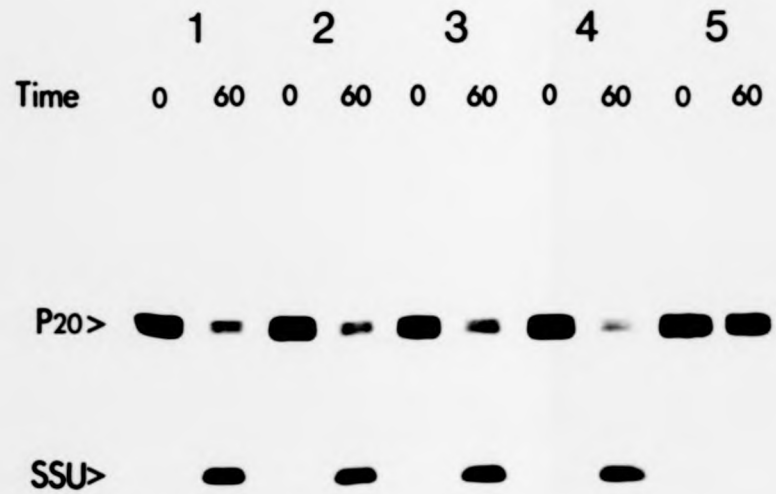
C. The effect of protease inhibitors on the processing enzyme

Despite catalysing the same reaction, proteases utilize widely differing mechanisms to cleave peptide bonds, giving rise to several classes of protease according to the functional groups involved in the reaction (Section I.4). A previously uncharacterised protease can be classified in this way by use of diagnostic inhibitors of each class. Serine proteases are generally inhibited by phenylmethylsulphonyl fluoride (PMSF), a compound which reacts with the catalytically-essential serine residue in the active site. The compounds N-ethylmaleimide and iodoacetate have a high affinity for cysteine thiol groups, and are therefore potent inactivators of thiol proteases. The reaction of these thiol reagents is essentially irreversible. The classical inhibitors of metalloproteases are the metal-chelating agents EDTA and 1,10-phenanthroline.

To date, no proteases have been isolated which contain a calcium atom in the active centre. However, a number of proteases (mainly from animal tissues) have been shown to be calcium-activated (Croall and De Martino, 1983). Several of these have been shown to be sensitive to the Ca^{2+}-chelating agent EGTA, and therefore this compound was tested for its effect on the processing enzyme.

The effects of these inhibitors on the processing of P20 by the purified processing enzyme is shown in Figure 16 and Table 7. Processing is inhibited markedly by EDTA and 1,10-phenanthroline, providing strong evidence that the enzyme is a metalloprotease. The enzyme is not affected by the serine- or thiol-protease inhibitors tested. It should


1 2 3 4 5
Time 0 60 0 60 0 60 0 60 0 60
P20>
SSU>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60
P20>
SSU>

Figure 16 The effect of protease inhibitors on the partially purified processing enzyme

Partially purified processing enzyme (Section II.9B) was incubated with in vitro-synthesised P20 (Section II.6D) in reaction mixtures of total volume 50 µl.

Incubation (1) was set up as described in Section II.10.

Incubation (2): as (1) but containing 5 mM EGTA.

Incubation (3): as (1) but containing 1 mM PMSF.

Incubation (4): as (1) except that the processing enzyme was first incubated with 10 mM iodoacetate for 60 min at 4°C, followed by dialysis overnight at 4°C against a large excess of 20 mM Tris-HCl, pH 7.6.

Incubation (5): as (1) but containing 5 mM 1,10-phenanthroline.

Immediately after addition of processing enzyme, a zero time sample (25 µl) was removed from each reaction mixture, mixed with one volume of 2 x sample buffer and boiled for 2 min. The remainder of each reaction mixture was incubated at 27°C for 60 min and then denatured as above. Samples were analysed by SDS polyacrylamide gel electrophoresis followed by fluorography (Sections II.7A and II.7C). SSU: authentic mature small subunit. Zero time (0 min) and 60 min samples from each incubation are indicated above the tracks.

Incubation 1 : control
Incubation 2 : + EGTA
Incubation 3 : + PMSF
Incubation 4 : pre-treatment with iodoacetate
Incubation 5 : + 1,10-phenanthroline.

Table 7 The effect of protease inhibitors on the processing of P20

Compound	Concentration mM	Inhibition of processing %
PMSF	1	0
EGTA	10	0
Iodoacetate	10	0
N-ethylmaleimide	10	0
1,10-phenanthroline	5	100
1,10-phenanthroline	1	69
1,10-phenanthroline	0.2	41
EDTA	10	94
EDTA	5	58

Processing enzyme was partially purified as described in Section II.9B and incubated with *in vitro*-synthesised P20 (Section II.6D) under conditions described in Section II.10. Processing was quantitated as described in Section II.7D.

The effect of N-ethylmaleimide was measured as for iododacetate (see legend to Fig. 16).

The effects of other inhibitors were measured as described in the legend to Fig. 16.

be pointed out that the free, unreacted thiol reagents were removed from the preparation of processing enzyme by dialysis after the incubation period; the reason for this is that the reaction of iodoacetate with P20 has a significant effect on the processing reaction (Section III.4A).

EGTA has no effect on the processing reaction, suggesting that Ca^{2+} ions play no role in regulating the activity of the protease.

D. Discussion

Protein chemical studies have shown that the processing enzyme is a metalloprotease of molecular weight approximately 180,000, with a pH optimum near 9.0 for the processing of P20.

The molecular weight is high for a protease; apart from leucine aminopeptidase, and a few others, the majority of proteases are small, monomeric enzymes of molecular weight 15,000 to 35,000 (Fersht, 1977). The high molecular weight of the processing enzyme suggests that the enzyme may be oligomeric, but if so, further purification and characterisation is required before the number, stoichiometry and function of the subunits can be established.

It should be emphasised that the pH-dependence of the whole processing reaction, and not strictly that of the activity of the processing enzyme, is shown in Figure 15. The ionisation and conformation of the substrate (P20) are presumably also affected by pH, giving rise to the possibility that the profile shown in Figure 15 represents an amalgam of the pH-dependence of enzyme activity and of substrate "acceptability".

The finding that the processing enzyme is inhibited by metal-chelators such as 1,10-phenanthroline, but not by serine- or thiol-protease inhibitors, provides strong evidence that the enzyme is a metalloprotease. With a number of metalloproteins it has proved possible to remove the essential metal atom(s) by prolonged dialysis against a solution containing a metal-chelator (such as 1,10-phenanthroline). Such a treatment has no apparent effect on the

processing enzyme; full activity is regained when the metal-chelator is removed by dialysis against fresh buffer, showing that the essential metal atom is tightly bound.

The data described in this section do not indicate the identity of the metal atom(s) involved in catalysis; both EDTA and 1,10-phenanthroline are capable of chelating a number of heavy metals. However, the chelator-sensitive metal is probably zinc since most metalloproteases have been found to contain this metal in the active centre. To identify the metal atom in the processing enzyme would require mass spectroscopy of milligram amounts of purified protein, which cannot yet be achieved (Section III.1C).

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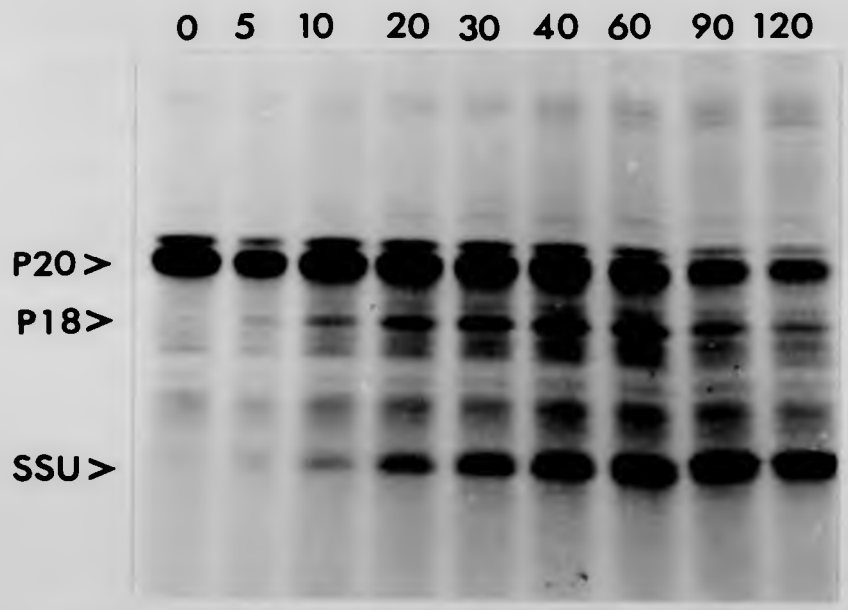
4. STUDIES ON THE MECHANISM OF THE SMALL SUBUNIT PRECURSOR PROCESSING REACTION

A. Two-step processing of the small subunit precursor

In experiments described previously in this thesis (e.g. Figure 3), and by Smith (1980), it was observed that processing of P20 led not only to the appearance of mature small subunit but also to the appearance of a polypeptide of molecular weight 18,000. In order to determine whether this polypeptide represented a processing intermediate or an *in vitro* artefact, a time course analysis of processing was performed (Figure 17). The 18,000 mol.wt. polypeptide appears during the course of the reaction, and subsequently disappears as the precursor is processed to mature small subunit, suggesting that processing of P20 involves two successive cleavages.

Further evidence of a two-step processing mechanism is provided by the finding that the second cleavage can be selectively inhibited. P20 which has been pre-incubated with iodoacetic acid is processed to the 18,000 mol.wt. form (P18), but no further, by the isolated processing enzyme (Figure 18). The accumulation of the P18 intermediate is due to reaction of iodoacetate with the precursor and not with the enzyme; the activity of processing enzyme is not affected by incubation with iodoacetate followed by removal of free reagent by dialysis (Section III.3C). Furthermore, if P20 is incubated with iodoacetate and then dialysed against processing buffer overnight to remove free reagent, the precursor is converted to the P18 form in subsequent processing


0 5 10 20 30 40 60 90 120
P20>
P18>
SSU>

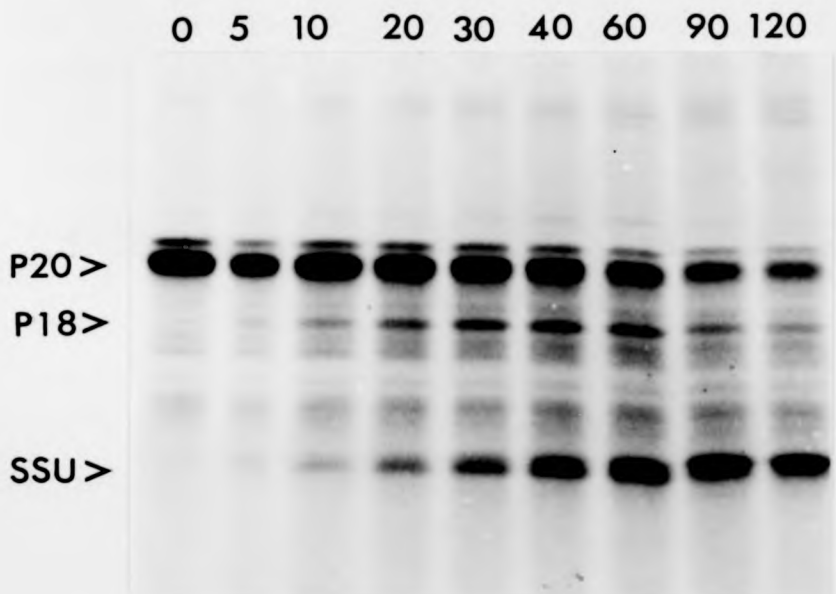
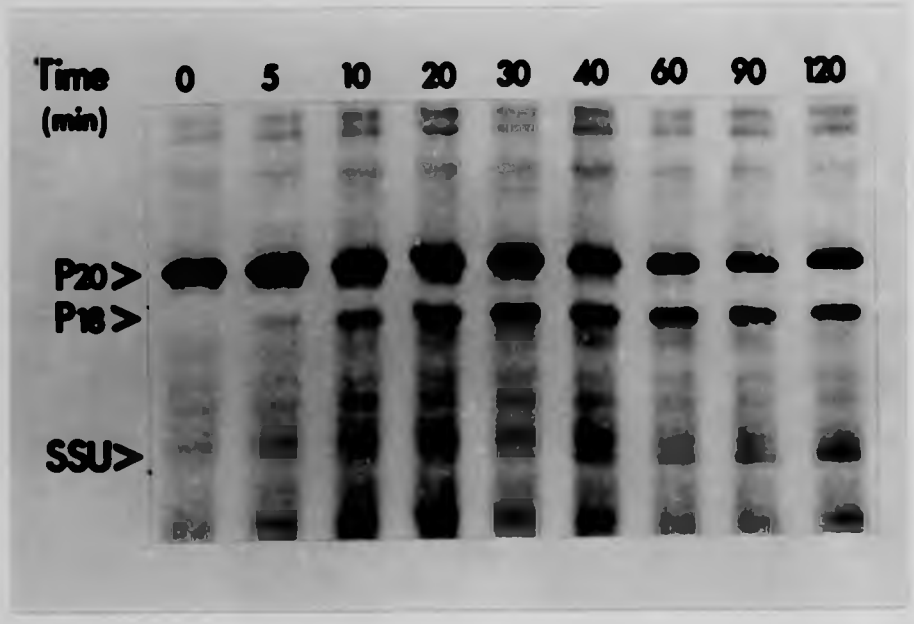

0 5 10 20 30 40 60 90 120
P20>
P18>
SSU>

Figure 17 Time course analysis of processing of P20 by the partially purified processing activity

Partially purified processing activity (Section II.9B) was incubated with *in vitro*-synthesised P20 (Section II) under conditions described in Section II.10. The incubation mixture consisted of 40 µl wheat-germ extract containing P20, 80 µl processing buffer and 80µl processing activity. Incubation was at 27°C. A sample (20 µl) was removed from the reaction mixture immediately after addition of the processing activity, and after incubation for 5, 10, 20, 30, 40, 60, 90 and 120 min. The samples were mixed with one volume of 2 x sample buffer and boiled for 2 min. A sample of wheat-germ extract containing P20 (4 µl) was diluted to 20 µl with water and denatured as above. Samples were analysed by SDS-polyacrylamide gel electrophoresis (Section II.7A) followed by fluorography (Section II.7C). SSU: authentic mature small subunit. P18: 18,000 mol.wt. polypeptide. Sample times are given above the tracks.


Time
(min)
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5
10
20
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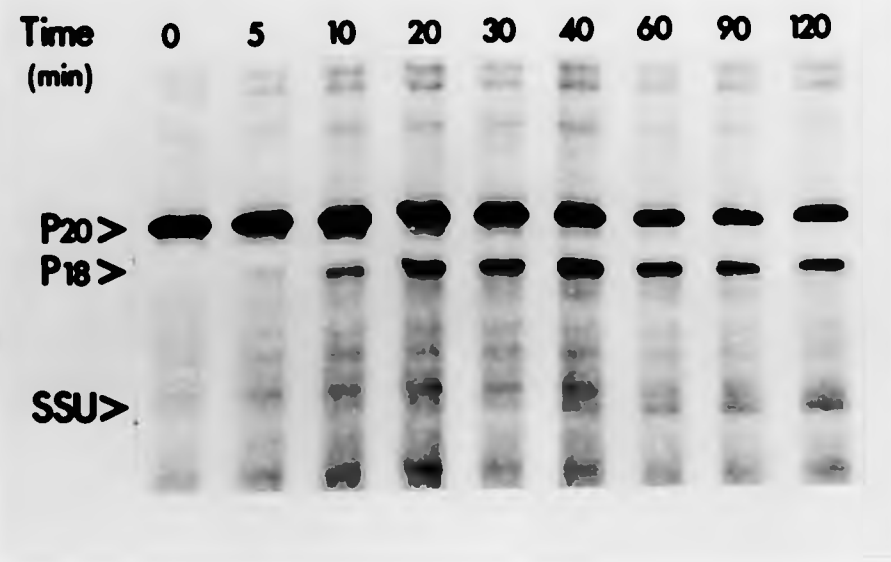
60
90
120
P20>
P18>
SSU>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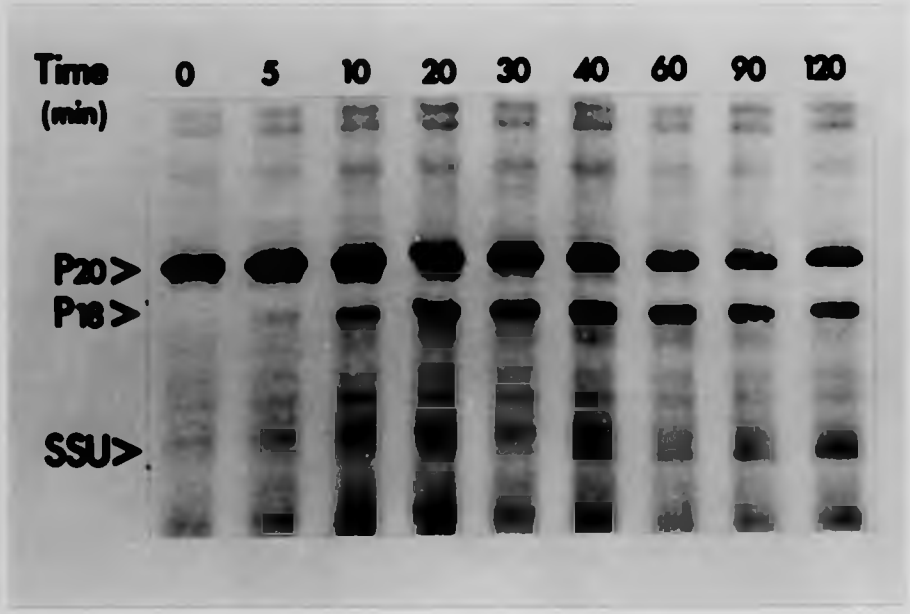
90
120
P20>
P18>
SSU>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P20>
P18>
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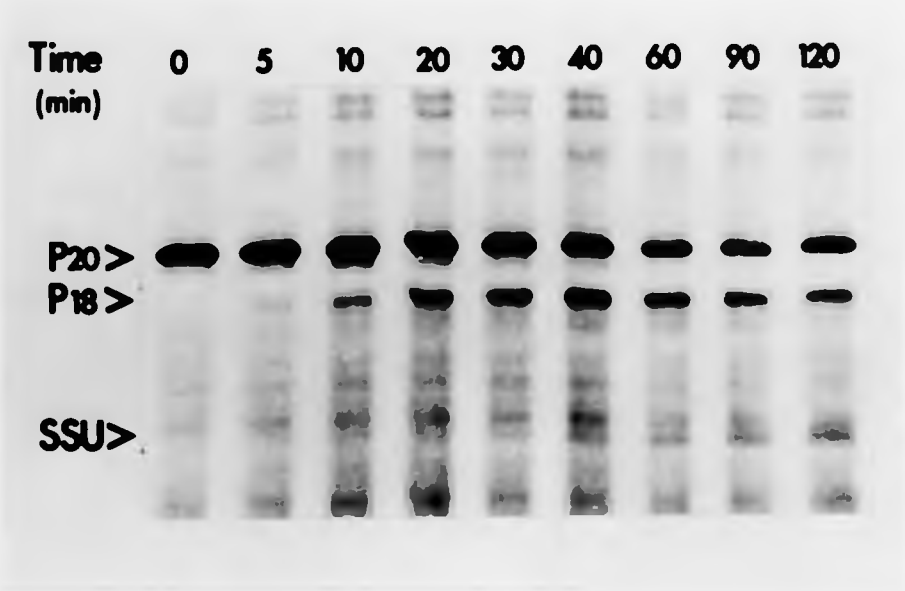
Time
(min)
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20
30
40
60
90
120
P20>
P18>
SSU>

Figure 18 Time course of processing of iodoacetate-treated P20

The time course analysis was performed exactly as described in Figure 17, except that the wheat-germ extract containing P20 (40 µl) was first mixed with 80 µl processing buffer containing 15 mM iodoacetic acid. After incubation for 30 min at 4°C, processing activity (80 µl) was added, the reaction mixture was incubated at 27°C, and samples were removed and analysed as described in Figure 17. SSU: mobility of authentic mature small subunit. P18: 18,000 mol.wt. polypeptide. Sample times are given above the tracks.

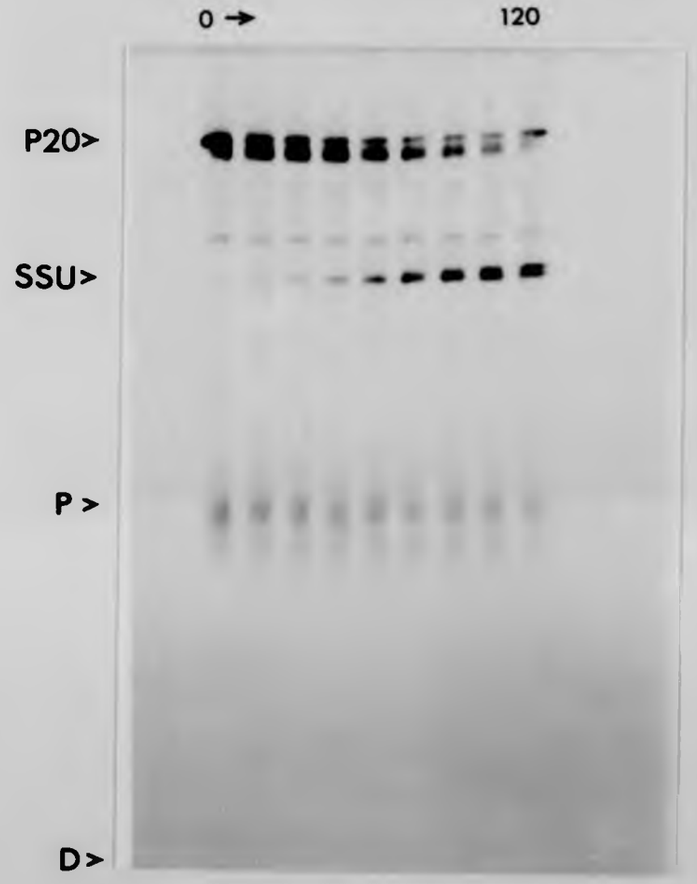
incubations (not shown).

The enzyme responsible for the processing of P20 to mature small subunit has been extensively purified (Section III.1C). The enzyme was purified approximately 300-fold by ammonium sulphate precipitation, gel filtration and ion-exchange chromatography. Other methods tested, but not used routinely, included hydroxyapatite chromatography and preparative non-denaturing gel electrophoresis (not shown). It has not been possible to separate different activities responsible for the two processing steps, and it is therefore highly likely that a single enzyme performs both cleavages. The fractionation of the processing enzyme using DEAE-Sephacel chromatography is illustrated in Figure 3; no fractions were produced which processed P20 to the P18 intermediate but no further.

In none of the processing experiments described in this or previous sections, has it been possible to identify any processed fragments on the fluorograms. The data described in this section suggest that the processing of P20 releases two fragments of molecular weight 2,000 and 4,000. It was considered possible that the failure to identify these peptides could be due to their size; peptides of such small size may have run off the gels in the experiments described so far. To test this possibility, a time-course analysis of processing was performed as described in Fig. 17, but the samples were resolved on a gel of greater length than usual (40 cm instead of 17 cm). The samples were loaded together with a blue dye, bromophenol blue, and electrophoresis was stopped before the dye reached the bottom of the gel. This procedure should ensure that no peptides are lost, because bromophenol blue

migrates with the buffer front. The results of this experiment are shown in Fig. 19. P20 is processed to small subunit but this reaction is not accompanied by the appearance of any peptides of low molecular weight. A peptide, or group of peptides (denoted P in Fig. 19) is present between the positions of the mature small subunit and the dye front (D), but the intensity of this band is identical in all of the tracks and therefore this band cannot represent the processed peptide(s).

The failure to observe processed peptides could be due to either of two reasons. Firstly, the peptides may be rapidly degraded by non-specific proteases in the wheat-germ translation mix, or secondly, the gel system used in this study may not be suitable for the resolution of small molecular weight peptides.


0 →
120
P20>
SSU>
P >
D>

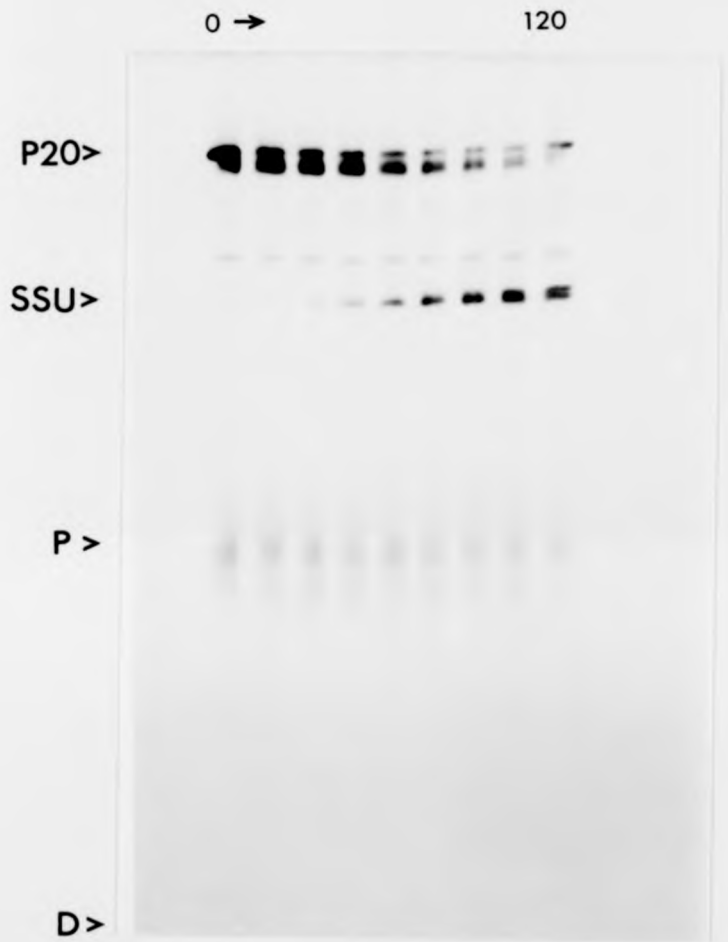

0 →
120
P20>
SSU>
P >
D>

Figure 19 Analysis of labelled low molecular weight peptides produced in the processing incubations

A time course analysis of processing of P20 was carried out exactly as described in Fig. 17. The samples were analysed by SDS polyacrylamide gel electrophoresis as described in Section II.7A except that the length of the gel was 40 cm instead of 17 cm. The samples were loaded on the gel together with the dye bromophenol blue and electrophoresis proceeded until the dye front (D) reached the bottom of the gel. The gel was fluorographed as described in Section II.7C. SSU, mobility of authentic mature small subunit; P, unidentified labelled peptides. Sample times from left to right are 0, 5, 10, 20, 30, 40, 60, 90 and 120 min.

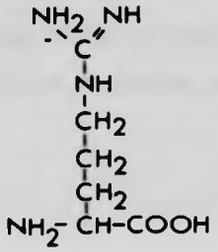
B. Processing of amino acid analogue-substituted small subunit precursor

A comparison of the extension sequences of small subunit precursor from pea, wheat and soybean reveals a number of common features. Several short regions of sequence homology are apparent, and the number and position of proline residues and positively charged amino acids is strongly conserved (Cashmore, 1983; Berry-Lowe et al., 1982). This raises the possibility that both three-dimensional structure (which proline residues influence considerably) and positive charges in the extension sequence are important for uptake into the organelle and/or processing. To test this possibility, amino acid analogues of proline and arginine were incorporaed into the P20 chain during synthesis in the wheat-germ system, and the abnormal precursors incubated with processing enzyme. This approach has been used to render preprolactin immune to processing during translocation across the endoplasmic reticulum (Hortin and Boime, 1981), and to inhibit Islet prohormone to hormone conversion (Noe, 1981). The amino acids used were azetidine-2-carboxylic acid (a four-membered ring analogue of proline) and canavanine (an arginine analogue). Due to differences in the bond angles in the azetidine carboxylate and proline rings, it has been postulated that azetidine carboxylate would rotate the polypeptide chain through an angle 15° less than would proline (Berman et al., 1969).

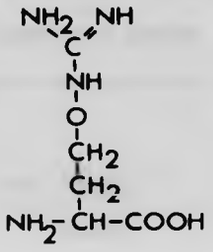
The pka of the guanidino group in arginine is above 12, and hence arginine residues are positively charged at physiological pH values. Canavanine contains instead a guanidoxy group, the pka of which is 7.4. The structures of these analogues are shown in Figure 20.

Figure 20 The structures of azetidine-2-carboxylic acid and canavanine

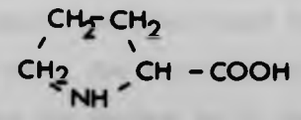
The structures of the amino acid analogues were taken from Data for Biochemical Research.



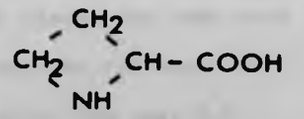
Arginine



Canavanine



Proline



Azetidine carboxylate

The results of incubating the processing enzyme with normal and analogue-substituted P20 are shown in Figure 21. Incorporation of either analogue markedly inhibits processing. Control experiments have established that the free forms of the analogues do not inhibit processing of normal P20 (not shown).

The incubations described in Figure 21 were carried out at pH 8.5; at this value 92.5% of the canavanine guanidoxy groups would be unprotonated according to the Henderson-Hasselbalch equation:

$$pH = pka + \log \frac{[RNH_2]}{[RNH_3^+]}$$

To test whether this loss of positive charge is the predominant factor in the low rate of processing of canavanine-substituted P20, processing incubations were carried out at pH 6.5, 7.5 and 8.5. The percentages of guanidoxy groups protonated at these pH values are 89, 45 and 7.5 respectively. The results are shown in Figure 22, and include control processing incubations of normal P20 to compensate for the effect of pH on the processing enzyme. It is apparent that the inhibitory effect of the canavanine residues is correlated with loss of positive charge; at pH 6.5, canavanine-substituted P20 is processed at almost the same rate as normal P20. The incorporation of canavanine into the P20 chain in fact lowers the optimum pH of the processing reaction from near 9.0 (Section III.3B) to around 7.5.

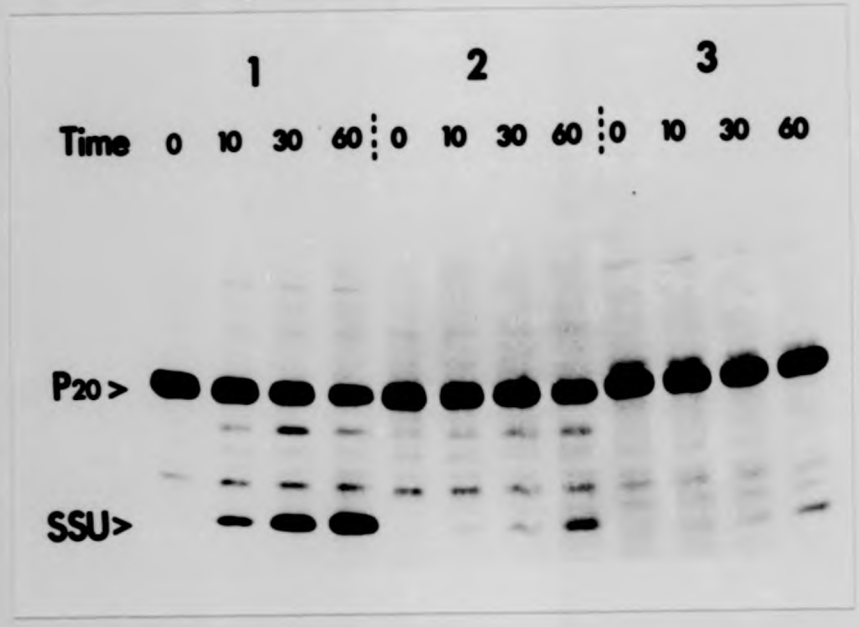

1
2
3
Time 0 10 30 60 0 10 30 60 0 10 30 60
P20 >
SSU>

Figure 21 Processing of analogue-substituted P20

Hybrid-released P20 mRNA (Section II.5) was translated in three wheat-germ incubations, each of total volume 20 µl. Incubation (1) was carried out as described in Section II.6D. Incubation (2) was carried out as in (1) except that proline was omitted from the reaction mixture and replaced by azetidine-2-carboxylic acid (final concentration 10 mM). Incubation (3) was carried out as in (1) except that arginine was omitted and replaced by canavanine (final concentration 10 mM). After incubation, 40 µl processing buffer (Section II.10) and 40 µl processing activity (Section II.9B) were added to each mixture. Each reaction mixture was incubated at 27°C. Samples were removed at zero time, 10 min, 30 min and 60 min, mixed with one volume of 2 x sample buffer, and boiled for 2 min. Samples were analysed by SDS polyacrylamide gel electrophoresis followed by fluorography. SSU: authentic mature small subunit. Sample times from each incubation are given above the tracks.

Figure 21 Processing of analogue-substituted P20

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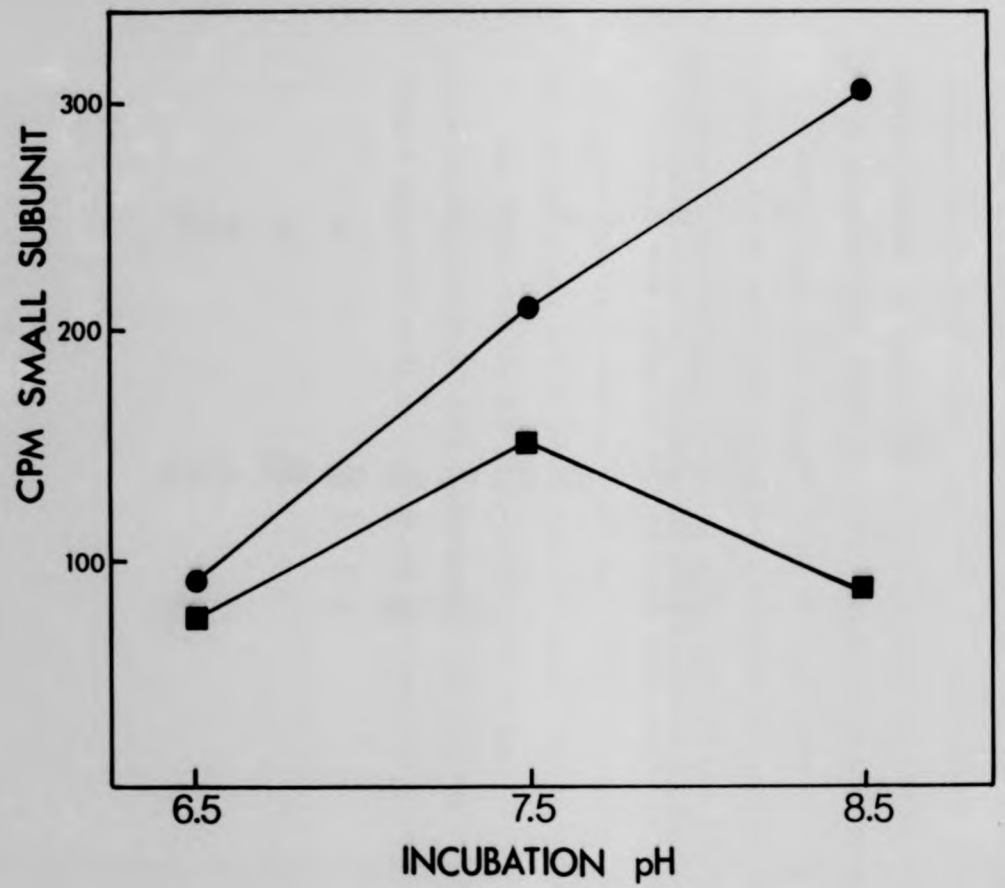
CPM SMALL SUBUNIT
300
200
100
6.5
7.5
8.5
INCUBATION pH

Figure 22 The pH-dependence of processing of canavanine-substituted P20

Aliquots (100 µl) of partially purified processing enzyme (Section II.9B) were dialysed overnight at 4°C against the following buffers: 300 mM HEPES-KOH, pH 6.5, 7.5 and 8.5. During the dialysis, P20 mRNA (Section II.5) was translated in two wheat-germ incubation mixtures of total volume 30 µl each. Incubation (1) was carried out as described in Section II.6D; incubation (2) was identical except that arginine was replaced by canavanine (10 mM). After incubation, each mixture was divided into three equal parts (A, B and C). Samples 1A and 2A were mixed with 20 µl processing buffer (Section II.10) in which the 100 mM HEPES-KOH, pH 8.5 component was replaced by 100 mM HEPES-KOH, pH 6.5. Samples 1B and 2B were mixed with 20 µl processing buffer containing 100 mM HEPES-KOH, pH 7.5. Samples 1C and 2C were mixed with 20 µl processing buffer. Processing enzyme (20 µl), dialysed as described above, was added to the incubations such that the pH of the preparation of processing enzyme matched that of the processing buffer in each incubation mixture. Each reaction mixture was incubated for 60 min at 27°C, then mixed with one volume of 2 x sample buffer and boiled for 2 min. Samples were analysed by SDS polyacrylamide gel electrophoresis followed by fluorography (Sections II.7A and II.7C). The extent of processing in each reaction was quantitated by excision of the labelled small subunit bands from the dried gel and measurement of [^{35}S] radioactivity (Section II.7D). The figure shows the pH-dependence of processing of normal (denoted ●-----●) and canavanine-substituted P20 (denoted ■-----■).

C. Discussion

The results presented in this section confirm the findings of Smith (1980) that processing of P20 leads to the appearance of polypeptides of molecular weights 14,000 (mature small subunit) and 18,000. This observation has been extended to show that the 18,000 mol.wt. polypeptide is an intermediate in a two-step processing mechanism. This is the first report of two-step processing of a cytoplasmically-synthesised chloroplast precursor polypeptide. Previous studies of the transport and processing of such precursors by isolated intact chloroplsts have been confined to a small number of proteins, and in these studies processing intermediates were not reported (Chua and Schmidt, 1978; Highfield and Ellis, 1978; Grossman *et al.*, 1982). Published data concerning the processing of precursors by chloroplast extracts are scarce. No intermediate was reported in the processing of small subunit precursor from *Chlamydomonas reinhardii* by a cell lysate (Dobberstein *et al.*, 1977), and none was apparent in the processing of wheat pre-plastocyanin described in this study (Section III.2A).

The enzymic activities responsible for the two cleavages co-chromatograph in all of the purification procedures tested so far, suggesting that a single processing enzyme performs both cleavages. This finding raises interesting questions regarding the specificity of the enzyme. The extension sequence of P20 from *Pisum sativum* has been partially (Bedbrook *et al.*, 1980) and fully (Cashmore, 1983) deduced from nucleotide sequencing of cloned DNA molecules from two varieties. With the exception of a single amino acid, the two sequences agree completely. The second cleavage takes place between a cysteine and a

methionine residue, methionine being the aminoterminal residue of the mature protein (Takruri et al., 1981). The site of the first cleavage is not known, but almost certainly neither cysteine nor methionine is involved in the scissile bond; the extension sequence contains only one cysteine residue, and none of the methionine residues are in the region of the extension sequence at which cleavage would yield a polypeptide of molecular weight about 18,000. The inhibition of the second, but not the first, processing step by pre-incubation of the precursor with iodoaceate may be due to this difference in the residues at the two cleavage sites. The carboxymethylation of the cysteine residue at the second site may mask the site from the processing enzyme, while the residues at the first cleavage site remain unaffected by iodoacetate. It should be pointed out that residues other than cysteine can be carboxymethylated by iodoacetate, though these reactions proceed much less readily (Gurd, 1967). Taken in conjunction with the data demonstrating the high reaction specificity of the processing enzyme (Section III.2) these results indicate that the enzyme recognises features of the precursor other than, or in addition to, the residues involved in the scissile bonds. The mode of action of the processing enzyme may therefore be radically different from the of the majority of "general" endoproteases; these proteases generally contain a specificity pocket, adjacent to the active centre, which preferentially binds the side chain of a particular amino acid or type of amino acid. The protease then cleaves the peptide bond on one side of the bound residue (Section I.4). The results with the processing enzyme do, however, suggest that the identity of the amino acids at the cleavage sites may be significant in that the presence of some residues may be unacceptable. It is possible that the inhibitory effect of iodoacetate

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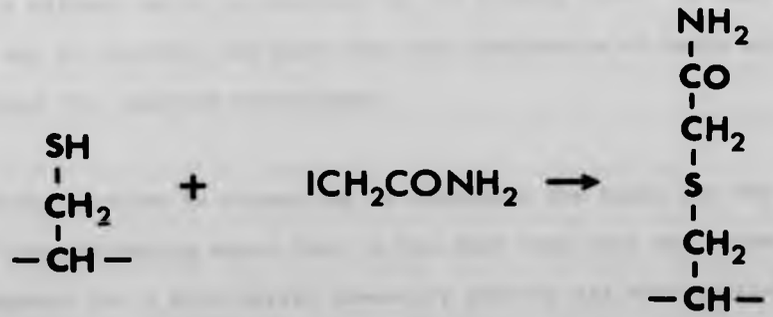
(or iodoacetamide) on the second cleavage reaction results solely from carboxymethylation of the cysteine residue at that site; this would imply that the formation of a bulkier side-chain has rendered the site immune to processing. The reaction of iodoacetate and iodoacetamide with the cysteine thiol group is shown below.



However, the inhibition could also conceivably result from carboxymethylation of the two cysteine residues in the mature sequence of the precursor, and, as pointed out above, residues other than cysteine can react with iodoacetate and iodoacetamide. Furthermore, it

should be emphasised that further purification of the processing activity is required in order to determine whether the activity comprises one or more species of enzyme.

In attempting to determine which features of the precursor are specifically recognised by the processing enzyme, two main possibilities should be considered. One is that the enzyme may recognise a specific residue or series of residues in the extension sequence, and then cleave the peptide bond a defined distance from this recognition site. The identities of the residues involved in the scissile bond may then be relatively insignificant, although certain types of side-chain may be unacceptable for steric reasons. A second possibility is that correct three-dimensional structure of the precursor may be essential for correct recognition and processing; the enzyme may recognise and bind a specific determinant of the precursor, and cleave the polypeptide chain at a defined point in relation to the binding site. In such a mechanism it may be possible for more than one combination of amino acids to create the required determinant.

A major problem in attempting to determine the basis for the specificity of the processing enyme lies in the fact that only one extension sequence for a chloroplast precursor protein has been published (that of the carboxylase small subunit). A knowledge of the extension sequence of other precursors (e.g. that of plastocyanin) is essential before the basis of processing enzyme-precursor recognition can be determined. In the absence of this vital information, two approaches can be used to address the problem using the sequence data available for the small subunit precursor.

The first approach is prompted by the finding that the pea precursor is processed in two steps, apparently by the same enzyme. Although the site of the first cleavage is not known, a prediction is that the features of the extension sequence that are recognised by the processing enzyme should be duplicated. An examination of the extension sequence of P20 reveals no sequence or pattern of amino acids in the aminoterminal half of the extension resembling that in the vicinity of the second cleavage site. It is not known whether there are duplicated regions of similar secondary structure that could serve as binding sites for the processing enzyme.

The second approach is to compare the extension sequences of small subunit precursor from pea, wheat and soybean. The lack of species-specificity of the processing enzyme (Section III.2) suggests that the enzyme may be capable of processing small subunit precursor from all three species, in which case the residues essential for recognition by the processing enzyme may be conserved in all three precursors. By the same argument, residues essential for recognition by the import machinery may also be conserved. As indicated earlier, the number and position of prolyl residues and positively-charged amino acids is strongly conserved, and the residues in the vicinity of the second cleavage site are similar in all three extension sequences. The importance of prolyl and arginyl residues in the precursor is evident from the results of experiments using analogues of these amino acids; substitution by azetidine carboxylate and canavanine virtually abolishes processing of the abnormal precursors. In the case of canavanine-substituted P20 the inhibition seems to stem from loss of positive charge. Taking into consideration the positions of prolyl and arginyl

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residues in the extension sequence of P20, these results can be explained in the following terms:

1. Substitution of proline by azetidine carboxylate

The positions of the two prolines in the extension sequence are a considerable number of residues from the second, and probably from the first, cleavage sites. It is therefore likely that the inhibition of processing results from one or both of the following effects:

i) alteration of specific secondary or tertiary structure in the extension sequence;

ii) alteration of overall tertiary structure due to substitution of proline residues in the mature part of the sequence.

2. Substitution of arginine by canavanine

The extension sequence in P20 contains three argininyl residues. One of these is located three residues from the second cleavage site, and the other two are in the vicinity of the first cleavage site. It is therefore possible that arginine residues are more directly involved in the specific recognition of the precursor by the processing enzyme than are prolyl residues. Possible explanations of the inhibitory effect of canavanine substitution include:

i) removal of essential positive charges near the cleavage sites that

are specifically recognised by the processing enzyme;

ii) alteration of essential three-dimensional structure in the extension sequence by removal of an essential salt-bridge(s);

iii) alteration of overall three-dimensional structure in the precursor by substitution of argininyl residues in the mature part of the sequence.

At present these possibilities can not be distinguished experimentally; the reasons for the inhibition of processing by analogue-substitution may become apparent when the extension sequences of a number of precursors have been deduced.

5. IMPORT AND PROCESSING OF SMALL SUBUNIT PRECURSOR BY ISOLATED INTACT CHLOROPLASTS

A. Introduction

A number of precursors, including that of the small subunit, have been used for studies on the mechanism of protein uptake by isolated intact chloroplasts (reviewed in Section I.3D). In none of these studies have any precursor molecules been detected inside the chloroplasts, giving rise to suggestions that the processing step may be an essential part of the import mechanism. The alternative explanation is that processing takes place very rapidly once the precursor has been imported into the organelle. However, it should be pointed out that in these studies, the failure to detect imported, unprocessed precursor molecules could be due to constraints on the experimental design. The majority of transport experiments have involved incubation of intact chloroplasts with poly(A)-enriched translation products, followed by electrophoretic analysis of the labelled, imported polypeptides present in the stroma and thylakoids. By far the most prominent labelled bands are those corresponding to the small subunit of RuBPCase and the chlorophyll a/b binding protein, and hence most import studies have concentrated on these polypeptides (discussed in detail in Section I.3D). However, whereas identification of these labelled, imported polypeptides is straightforward, the presence of a large number of additional labelled bands precludes identification of any unprocessed precursor molecules among the imported polypeptides. Furthermore, immunoprecipitation of the labelled polypeptides is made difficult by the presence of large

quantities of the unlabelled proteins; in a typical import assay such as that described in Section II.11, the sample of chloroplasts contains approximately 150 µg RuBPCase and 50 µg chlorophyll a/b binding protein. For these reasons, none of the previous published import studies have seriously attempted to measure the delay, if any, between transport and processing.

The availability of purified P20 mRNA allows a more satisfactory analysis of the import process to be carried out. Incubation of intact chloroplasts with in vitro-synthesised P20 would be expected to lead to the appearance of labelled, mature small subunit in the stroma. If processing takes place during, or very shortly after, transport into the organelle, no other labelled polypeptide should be apparent. Alternatively, the presence of imported, unprocessed P20 would show that processing takes place some time after the transport step, and that processing is not an obligatory part of the transport system.

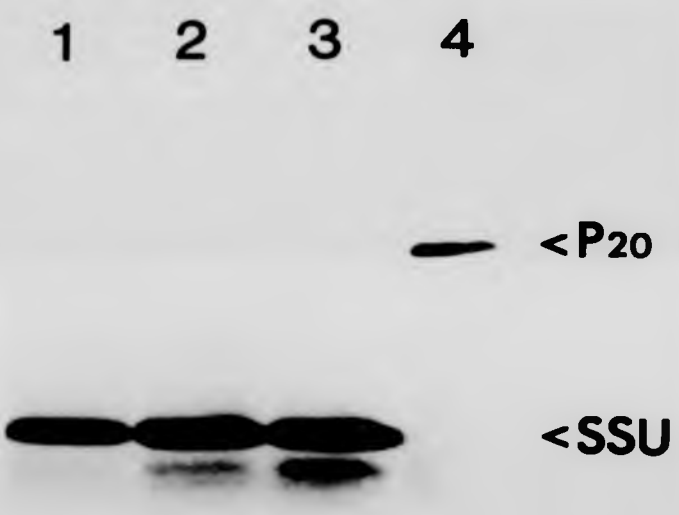
Studies on the isolated processing enzyme described earlier in this thesis have shown that the in vitro processing of P20 can be inhibited by three methods. These include the use of metal-chelating agents (Section III.3C), incorporation of amino acid analogues into the P20 chain (Section III.4B) and the selective inhibition of the second processing step by pre-incubation of P20 with iodoacetate (Section III.4A). This section describes experiments designed to test the effects of these methods of inhibition on the uptake and processing of P20 by isolated intact chloroplasts.

The uptake assay used was modified from that of Chua and Schmidt (1978), and involved incubation of *in vitro*-synthesised P20 or poly(A)-containing RNA translation products with purified, intact chloroplasts under illumination (particulars are given in Section II.11). At the end of the incubation period the mixture was placed on ice and incubated with trypsin to degrade polypeptides outside the chloroplasts. At the same time, 1,10-phenanthroline was added to prevent processing of any precursor molecules inside the chloroplasts; 1,10-phenanthroline is a potent inhibitor of the isolated processing enzyme (Section III.3C), and is capable of entering chloroplasts and inhibiting chloroplast protein synthesis (unpublished). After the protease treatment the chloroplasts were lysed, and the stromal polypeptides analysed by SDS polyacrylamide gel electrophoresis followed by fluorography.

B. Results

In order to determine whether P20 or P18 can be detected inside intact chloroplasts, a time course analysis of the uptake process was performed. The results are shown in Fig. 23. P20 is rapidly taken up by the chloroplasts and converted to mature small subunit. Despite the 1,10-phenanthroline treatment at the end of the incubation period, no P20 or P18 can be detected inside the chloroplasts, showing that both processing steps take place either during transport into the organelle or shortly afterwards.

A similar time course analysis was performed using P20 which had been pre-incubated with iodoacetate to block the second processing step. Fig. 24 shows that the precursor is taken up and converted to a number of polypeptides of molecular weight 18,000 and below. The number of bands is unexpected; one possible explanation is that the "ladder" of bands is due to an effect of iodoacetate entering the chloroplasts. This possibility was tested by omitting unlabelled methionine from the uptake incubation mixture; free [^{35}S]-methionine in the wheat-germ extract is then taken up by the chloroplasts and incorporated into protein by the chloroplast protein synthetic system. The presence of iodoacetate inside the chloroplasts would be expected to inhibit this incorporation, either by a direct effect on the protein synthetic system or by reacting with all of the available cysteine. However, chloroplast protein synthesis proceeds normally when the uptake mixture contains iodoacetate, suggesting that the reagent does not enter the organelle (Fig. 25, cf tracks 2 and 3). The labelled stromal polypeptides in track 2 comprise imported carboxylase small subunit plus a number of


1
2
3
4
<P20
<SSU

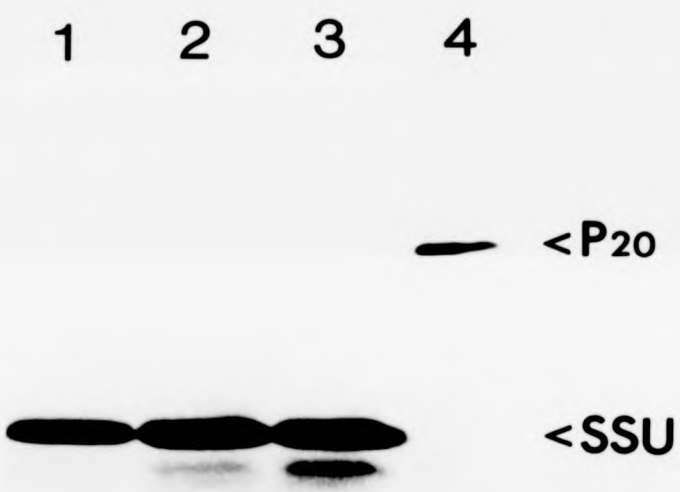

1
2
3
4
<P20
<SSU

Figure 23 Uptake of P20 by intact isolated chloroplasts

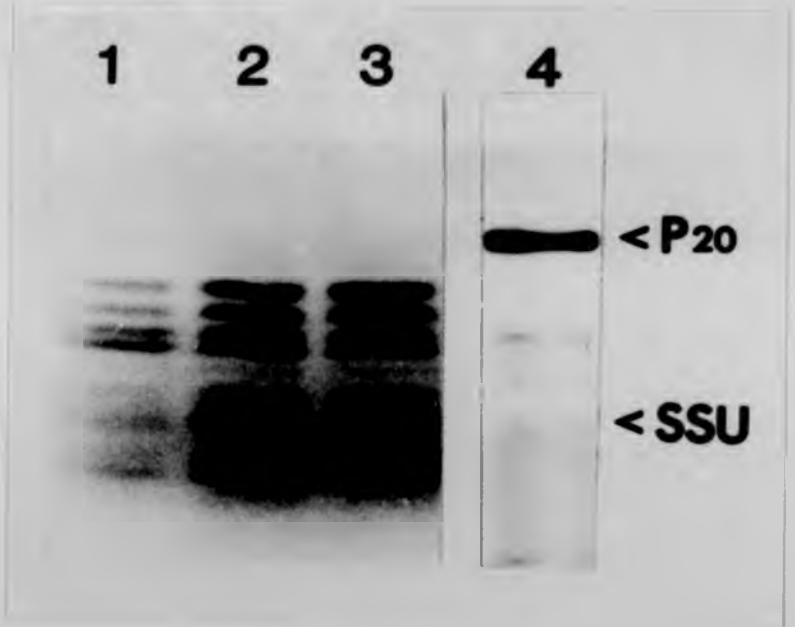
Uptake assays were carried out as described in Section II.11. Three incubation mixtures were set up, each containing 100 µl purified intact chloroplasts (90 µg chlorophyll) and 10 µl wheat-germ extract containing P20 (Sections II.8B and II.6D), together with the other components detailed in Section II.11. After incubation at 25°C, under illumination, for 8, 20 and 60 min the mixtures were transferred into ice and incubated with trypsin (1 mg/ml) and 1,10-phenanthroline (25 mM) for 30 min at 4°C. The chloroplasts were then re-isolated, lysed, and the labelled, stromal polypeptides analysed by SDS polyacrylamide gel electrophoresis followed by fluorography (Sections II.7A and II.7C). SSU: authentic mature small subunit.

Track (1) in vitro-synthesised P20

(2) 8 min incubation, stromal fraction

(3) 20 min incubation, stromal fraction

(4) 60 min incubation, stromal fraction


1
2
3
4
< P20
< SSU

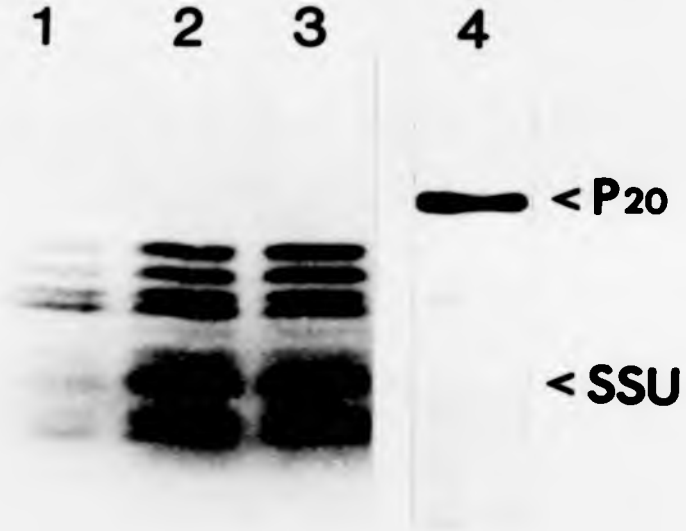
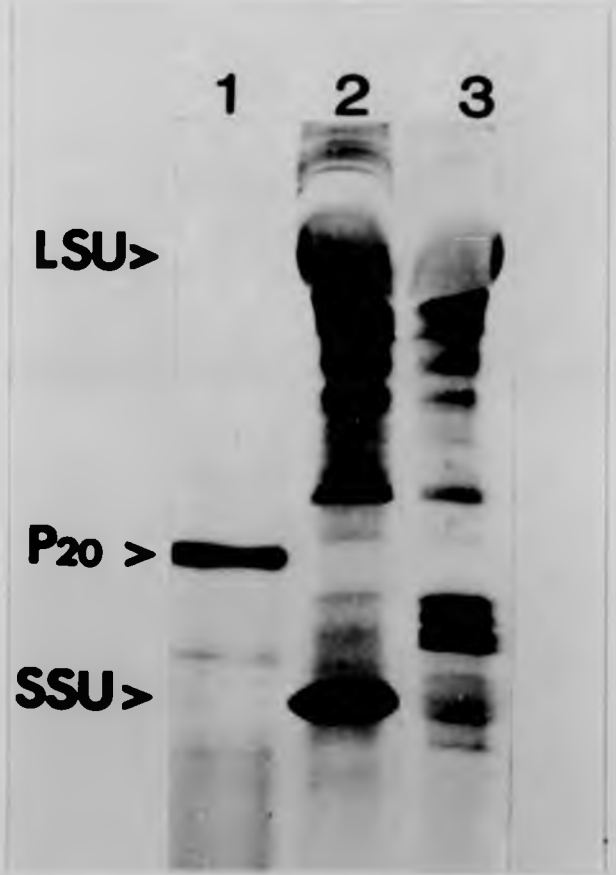

1
2
3
4
< P20
< SSU

Figure 24 Uptake of iodoacetate-treated P20 by intact isolated chloroplasts

Uptake assays were set up as described in Section II.11 except that the SRM component contained 20 mM iodoacetic acid. The SRM/iodoacetate (10 µl) was pre-incubated with the wheat-germ extract containing P20 (10 µl) for 30 min at 4°C before the other components of the uptake mixture were aded (i.e. methionine, 5 x SRM, and purified, intact chloroplasts). Three uptake assays were carried out. After incubation for 8, 20 and 60 min, the mixtures were transferred into ice and incubated with trypsin and 1,10-phenanthroline for 30 min at 4°C as described in Section II.11. The chloroplasts were then re-isolated, lysed and the labelled, stromal polypeptides analysed by SDS polyacrylamide gel electrophoresis followed by fluorography (Sections II.7A and II.7C). SSU: authentic mature small subunit.

Track (1) 8 min incubation, stromal fraction
(2) 20 min incubation, stromal fraction
(3) 60 min incubation, stromal fraction
(4) *in vitro* synthesised P20


1
2
3
LSU>
P20 >
SSU>

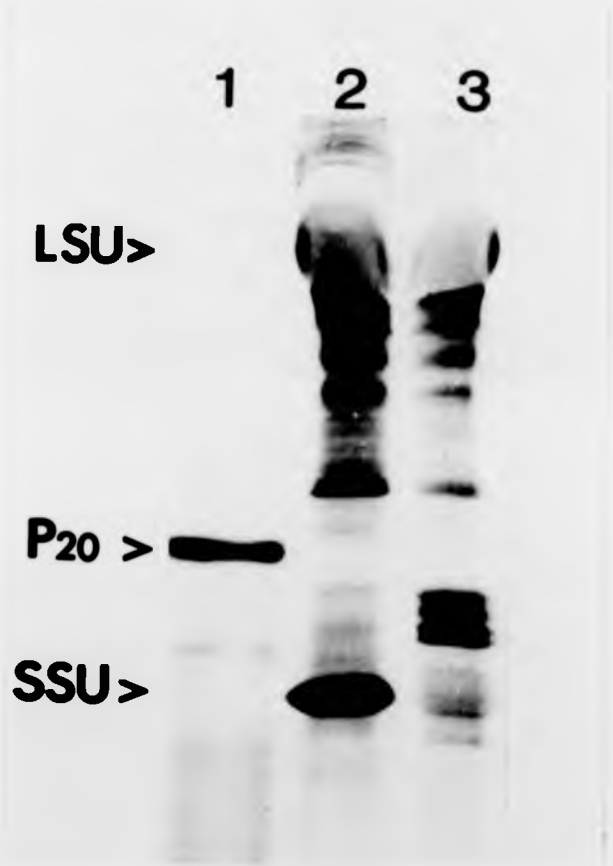

1
2
3
LSU>
P20 >
SSU>

Figure 25 Chloroplast protein synthesis during the uptake of untreated and iodoacetate-treated P20 by intact isolated chloroplasts

Uptake assays were carried out as described in Section II.11 except where specified.

Incubation (1): *in vitro* synthesised P20 was incubated with intact isolated chloroplasts, together with the other components detailed in Section II.11, except that unlabelled methionine was omitted and replaced with 50% (v/v) SRM. Incubation was for 20 min under illumination.

Incubation (2): as (1) except that the P20 was pre-treated with iodoacetate as described in Fig. 24.

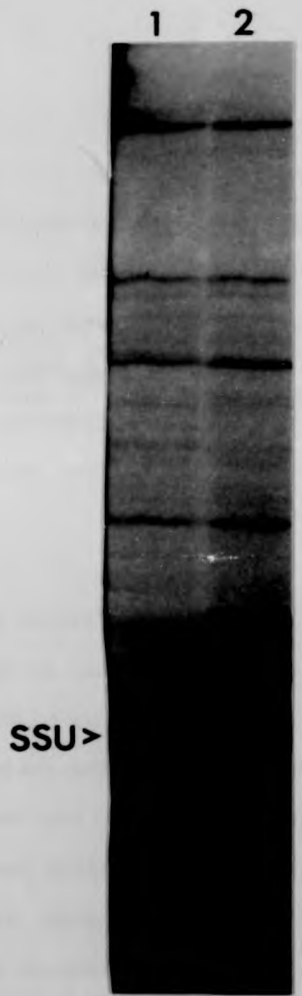
After incubation, the chloroplasts were trypsinised, re-isolated, lysed, and the labelled stromal polypeptides analysed by SDS polyacrylamide gel electrophoresis followed by fluorography (Sections II.7A and II.7C).

Track 1 : *in vitro*-synthesised P20
Track 2 : incubation (1), stromal fraction
Track 3 : incubation (2), stromal fraction

chloroplast translation products, including the large subunit of ribulose bisphosphate carboxylase. The same chloroplast translation products are apparent in track 3 together with the ladder of bands due to import of iodoacetate-treated P20.

Another possible explanation for the number of bands is that they arise because of incomplete removal of trypsin from the reaction mixture. This was tested by using Staphylococcus aureus V8 protease instead of trypsin to degrade non-imported proteins. This enzyme cleaves proteins at acidic residues, in contrast to trypsin which cleaves at basic residues. In this experiment, P20 was imported and converted to the same ladder of bands as that observed in Fig. 24, suggesting that the appearance of the bands is not due to the action of the commercial protease preparation (data not shown).

The results described in this Section and in Section III.4, show that the carboxymethylation of one or more cysteine residues in the P20 chain inhibits processing at the second cleavage site. It is highly likely that this effect is due to modification of the cysteine residue involved in the scissile bond at the second cleavage site. To test the possibility that the carboxymethylation of other imported precursors affects transport or processing of the modified molecules, the import of carboxymethylated poly(A)-enriched RNA translation products was studied. Figure 26 shows the results of incubation of intact chloroplasts with untreated and iodoacetate-treated poly(A)-enriched RNA translation products. Apart from the appearance of the ladder of bands resulting from import and partial processing of carboxymethylated P20, the number, mobility and intensity of the other bands in the two tracks are very


1
2
SSU>


1
2
SSU>

Figure 26 Uptake of iodoacetate-treated poly(A)-enriched RNA translation products by intact isolated chloroplasts

Uptake assays were carried out as described in Section II.11 except where changes are specified. Two incubation mixtures were set up, each containing 100 µl purified intact chloroplasts (Section II.8B) and 10 µl wheat-germ extract containing pea leaf poly(A)-enriched RNA translation products (Section II.6C), together with the other components detailed in Section II.11.

Incubation (1): incubation period was 20 min, after which the chloroplasts were trypsinised, re-isolated, lysed, and the stromal polypeptides analysed by SDS polyacrylamide gel electrophoresis and fluorography (Sections II.7A and II.7C).

Incubation (2): as (1) except that the poly(A)-enriched RNA tranlation products were treated with iodoacetate (as described in Fig. 24 for P20) before addition of the other components of the reaction mixture.

SSU: authentic mature small subunit.

Track (1) : incubation (1), stromal fraction

Track (2) : incubation (2), stromal fraction

similar, indicating that iodoacetate treatment has no effect on the transport and processing of the majority of imported polypeptides.

The effect of incorporation of amino acid analogues on the import of P20 by isolated chloroplasts was tested using the same uptake assay. The experiment also examined the effect of the metal-chelating agents 1,10-phenanthroline and bathophenanthroline disulphonate on the uptake and processing of normal P20. (Bathophenanthroline disulphonate is a more water-soluble derivative of 1,10-phenanthroline). The results are shown in Fig. 27. In the control incubation (track 2), P20 is taken up by the chloroplasts and processed to mature small subunit. In the presence of 1,10-phenanthroline (track 3) or bathophenanthroline disulphonate (track 4) the rate of uptake is markedly reduced. Similarly, azetidine carboxylate-substituted P20 is taken up at a much-reduced rate (track 5). Canavanine-substituted P20 is taken up at approximately 20% of the control rate (track 6). No imported, unprocessed precursor molecules are apparent in tracks 2-6, consistent with the possibility that processing may be an essential part of the import mechanism.

In order to study the effect of metal-chelators and analogue-substitution on the import of other cytoplasmically-synthesised chloroplast polypeptides, the type of experiment shown in Fig. 27 was repeated using poly(A)-containing RNA translation products instead of hybrid-released P20 mRNA translation products. The effects of 1,10-phenanthroline and bathophenanthroline disulphonate are shown in Fig. 28. The presence of either metal-chelator significantly inhibits import of stromal (Fig. 28A) and thylakoidal (Fig. 28B) polypeptides.

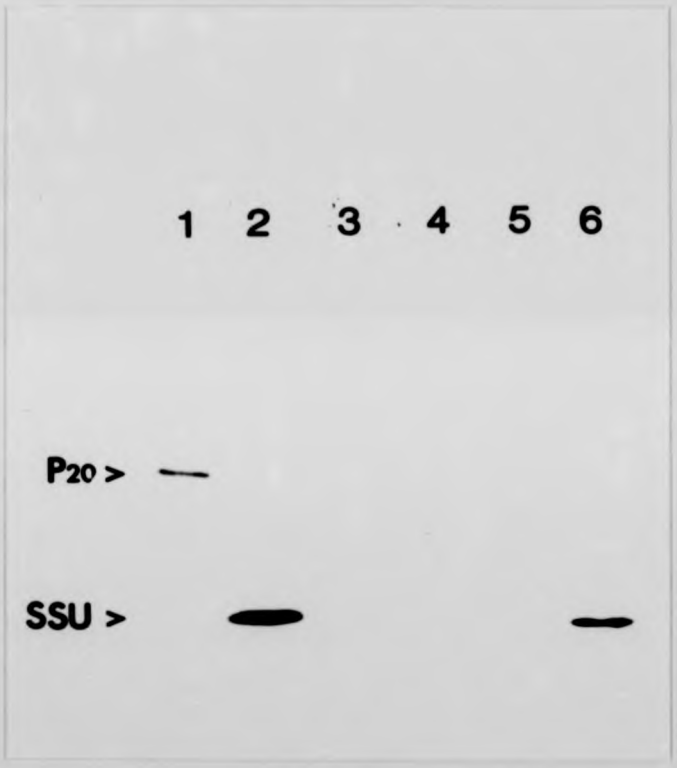

1 2 3 4 5 6
P20 >
SSU >

Figure 27 The effect of processing inhibitors on the uptake and processing of P20 by intact isolated chloroplasts

Uptake assays were carried out as described in Section II.11, except where additions are specified.

Incubation mixture (1): contained *in vitro*-synthesised P20, intact isolated chloroplasts and other components as detailed in Section II.11.

Incubation mixture (2): as (1) but containing 3 mM 1,10-phenanthroline.

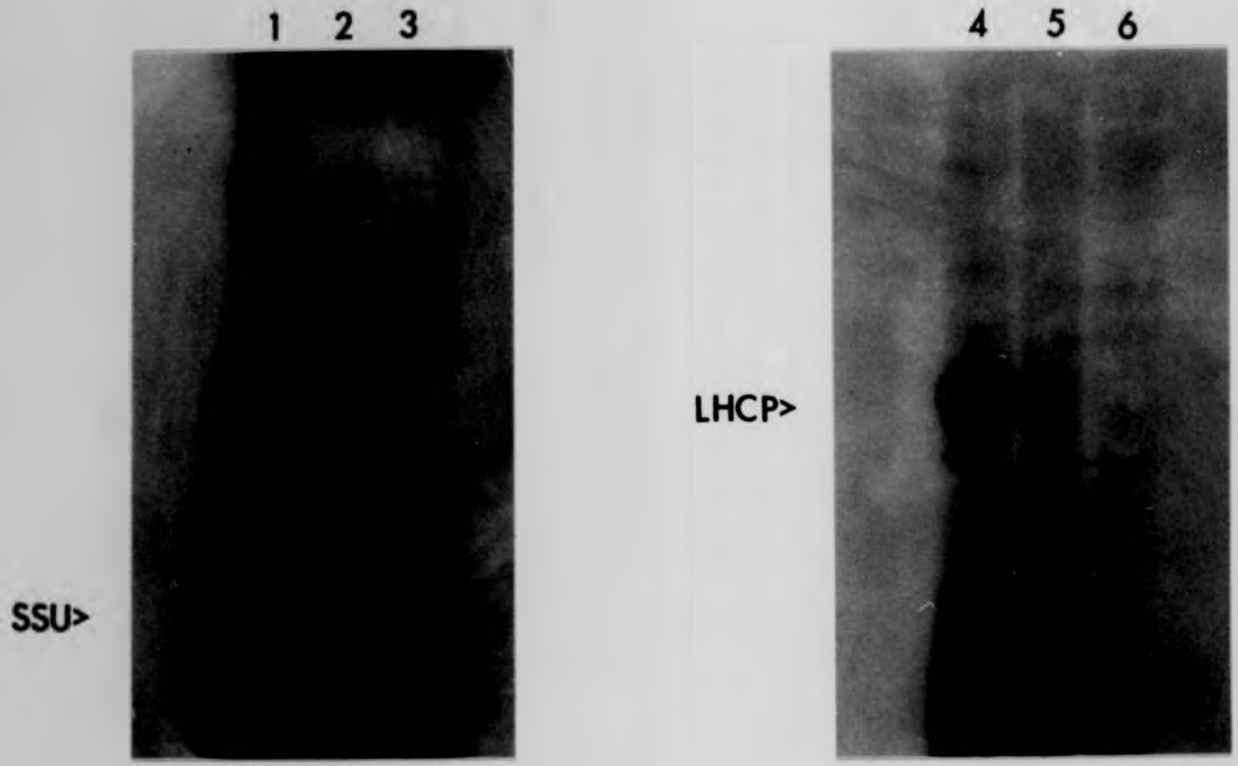
Incubation mixture (3): as (1) but containing 6 mM bathophenanthroline disulphonate.

Incubation mixture (4): as (1) except that P20 was synthesised in the presence of azetidine carboxylate in place of proline as described in the legend to Fig. 21.

Incubation mixture (5): as (1) except that P20 was synthesised in the presence of canavanine in place of arginine.

Incubation was for 5 min under illumination, after which the stromal polypeptides were analysed by SDS polyacrylamide gel electrophoresis followed by fluorography (Sections II.7A and II.7C). SSU: authentic mature small subunit.

Track 1 : *in vitro*-synthesised P20
Track 2 : control incubation
Track 3 : + 1,10-phenanthroline
Track 4 : + bathophenanthroline disulphonate
Track 5 : uptake of azetidine carboxylate substituted P20
Track 6 : uptake of canavanine substituted P20


1 2 3
4 5 6
SSU>
LHCP>

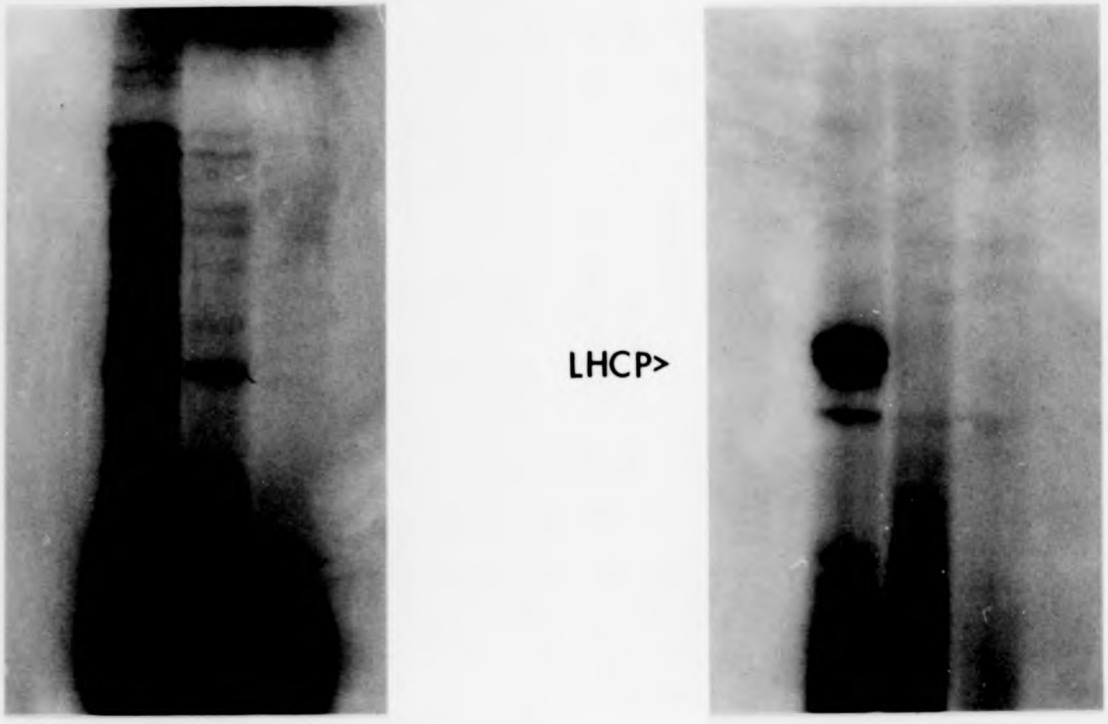

1 2 3
4 5 6
SSU>
LHCP>

Figure 28 The effect of metal-chelators on the uptake of poly(A)-enriched RNA translation products by intact isolated chloroplasts

Uptake assays were carried out as described in Section II.11, except where additions are specified.

Incubation mixture (1): contained *in vitro*-synthesised pea leaf poly(A)-enriched RNA translation products, intact isolated chloroplasts and other components as detailed in Section II.11.

Incubation mixture (2): as (1) but containing 6 mM bathophenanthroline disulphonate.

Incubation mixture (3): as (1) but containing 3 mM 1,10-phenanthroline.

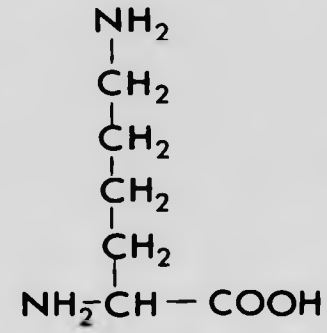
Incubation was for 20 min under illumination, after which the chloroplasts were trypsinised, re-isolated and lysed. The lysates were centrifuged and the stromal supernatants removed, mixed with one volume of 2 x sample buffer (Section II.7A) and boiled for 2 min. The thylakoid pellets were washed twice with 400 µl 20 mM Tris-HCl, pH 7.6 and then resuspended in 100 µl of a 1:1 mixture of 20 mM Tris-HCl, pH 7.6 and 2 x sample buffer, followed by boiling for 2 min. Samples were analysed by SDS polyacrylamide gel electrophoresis followed by fluorography (Sections II.7A and II.7C). SSU: authentic mature small subunit; LHCP: light harvesting chlorophyll a/b binding protein.

Track 1 : control incubation, stromal fraction,
Track 2 : + bathophenanthroline disulphonate, stromal fraction
Track 3 : + 1,10-phenanthroline, stromal fraction
Track 4 : control incubation, thylakoid fraction
Track 5 : + bathophenanthroline disulphonate, thylakoid fraction
Track 6 : + 1,10-phenanthroline, thylakoid fraction

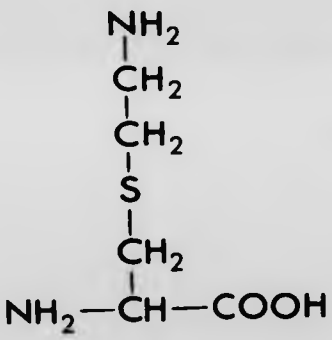
The effect of substitution of proline and lysine residues on the import of stromal and thylakoidal polypeptides was investigated by incorporation of the amino acid analogues azetidine carboxylate and thialysine into poly(A) containing RNA translation products. The inhibition of import of azetidine carboxylate-substituted P20 has been described in Fig. 27. Incorporation of thialysine, a lysine analogue, has also been found to inhibit processing of P20 by the partially purified processing enzyme and import of P20 into isolated intact chloroplasts (data not shown). The structure of thialysine is shown in Fig. 29. The effect of thialysine or azetidine carboxylate substitution on the import of poly(A)-enriched RNA translation products is shown in Fig. 30. If the fluorogram representing stromal polypeptides is exposed to X-ray film for a short period of time (overnight) it is observed that incorporation of either analogue markedly inhibits the import of P20 (Figure 30A, cf tracks 1, 4 and 7). The effect of analogue-substitution on the import of other, less prominent stromal polypeptides is shown by exposure of the same gel for 14 days (Fig. 30B); a number of these polypeptides are imported at much-reduced rates. Overall, substitution of proline residues by azetidine carboxylate inhibits the import of more polypeptides that does substitution of lysine residues by thialysine. However, it is also apparent that the import of some polypeptides is not affected by analogue-substitution.

The imported thylakoid polypeptides from the same experiment are shown in Fig. 30C. Again, incorporation of thialysine or azetidine carboxylate inhibits the import of a number of polypeptides. In particular, import of the light-harvesting chlorophyll a/b binding protein (LHCP) is almost completely abolished by the incorporation of

Figure 29 The Structure of Thialysine



Lysine

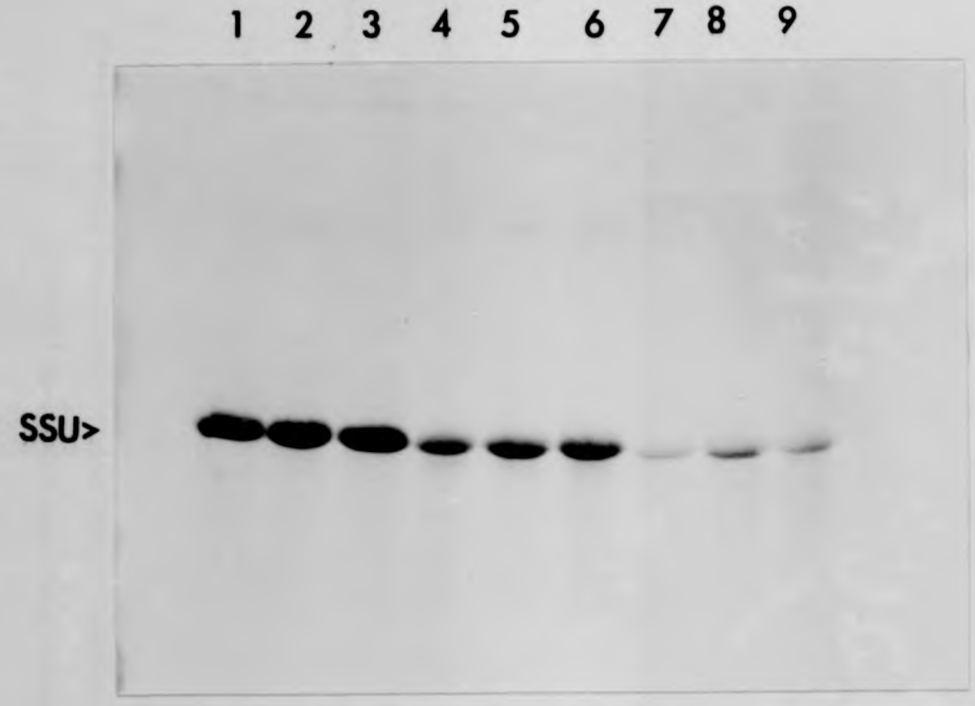


Thialysine

Figure 30 The effect of analogue substitution on the uptake of poly(A)-enriched RNA translation products by intact isolated chloroplasts

Pea leaf poly(A)-enriched RNA (SectionII.3B) was translated in three wheat-germ incubation mixtures of 30 µl each. Incubation mixture (1) was set up as described in SectionII.6C. Incubation mixture (2) was identical except that lysine in the reaction mixture was replced by 10 mM thialysine. Incubation mixture (3) was as (1) except that proline in the reaction mixture was replaced by 10 mM azetidine carboxylate. After translation, each incubation mixture was divided into three equal aliquots, and each aliquot was mixed with intact isolated chloroplasts under the conditions described in Section II.11. Incubations were carried out for 8 min, 20 min and 40 min for each of the wheat-germ incubation mixtures (1), (2) and (3). After incubation, each sample of chloroplasts was trypsinised, re-isolated, lysed and centrifuged as described in Section II.11. Each stromal supernatant was removed, mixed with one volume of 2 x sample buffer (Section II.7A) and boiled for 2 min. The thylakoid pellets were washed twice with 400 µl 20 mM Tris-HCl, pH 7.6 and then resuspended in 50 µl of the same buffer. Each sample was mixed with one volume of 2 x sample buffer and boiled for 2 min. Samples were analysed by SDS polyacrylamide gel electrophoresis followed by fluorography (Sections II.7a and II.7C).

SSU: authentic mature small subunit; LHCP: mature light harvesting chlorophyll a/b binding protein.


1 2 3 4 5 6 7 8 9
SSU>

A. Stromal Fractions. The dried gel was exposed to X-ray film overnight.

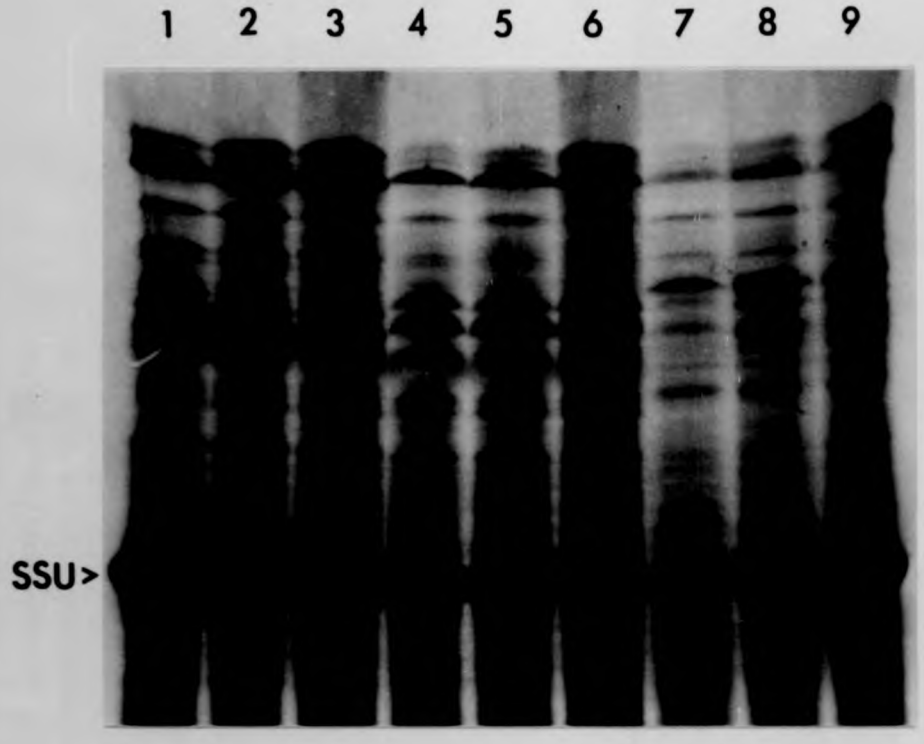
Track 1 : control incubation - 8 min
Track 2 : control incubation - 20 min
Track 3 : control incubation - 40 min
Track 4 : Thialysine incorporation - 8 min
Track 5 : Thialysine incorporation - 20 min
Track 6 : Thialysine incorporation - 40 min
Track 7 : Azetidine carboxylate incorporation - 8 min
Track 8 : Azetidine carboxylate incorporation - 20 min
Track 9 : Azetidine carboxylate incorporation - 40 min

B. Stromal Fractions. The dried gel was exposed to X-ray film for 14 days. Tracks are as in A.

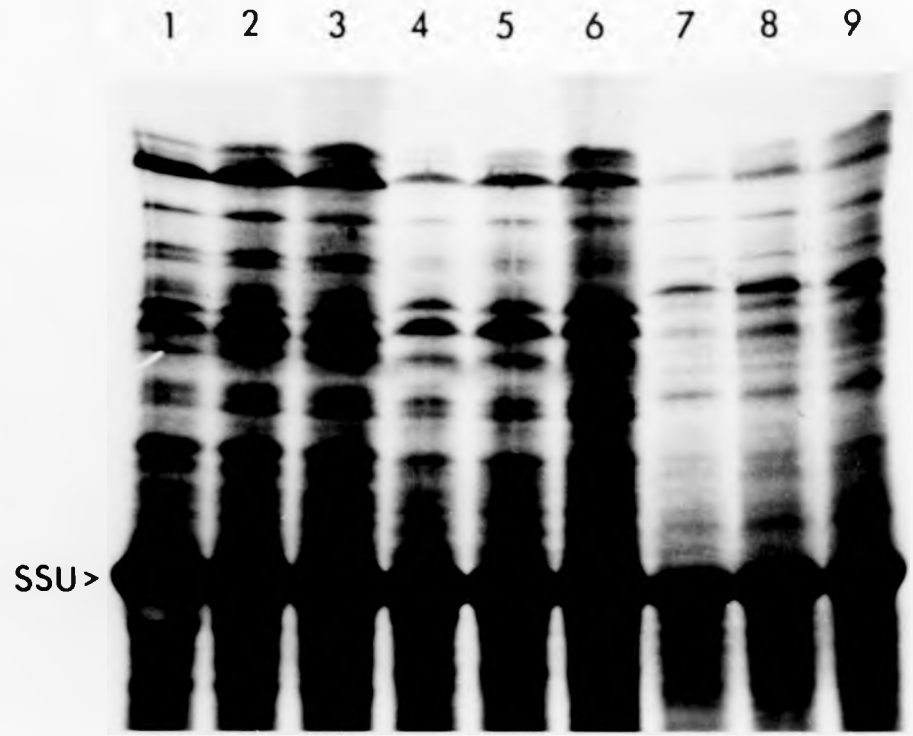
Track 1 : control incubation - 8 min
Track 2 : control incubation - 20 min
Track 3 : control incubation - 40 min
Track 4 : Thialysine incorporation - 8 min
Track 5 : Thialysine incorporation - 20 min
Track 6 : Thialysine incorporation - 40 min
Track 7 : Azetidine carboxylate incorporation - 8 min
Track 8 : Azetidine carboxylate incorporation - 20 min
Track 9 : Azetidine carboxylate incorporation - 40 min

C. Thylakoid fractions. The dried gel was exposed to X-ray film for 30 days.

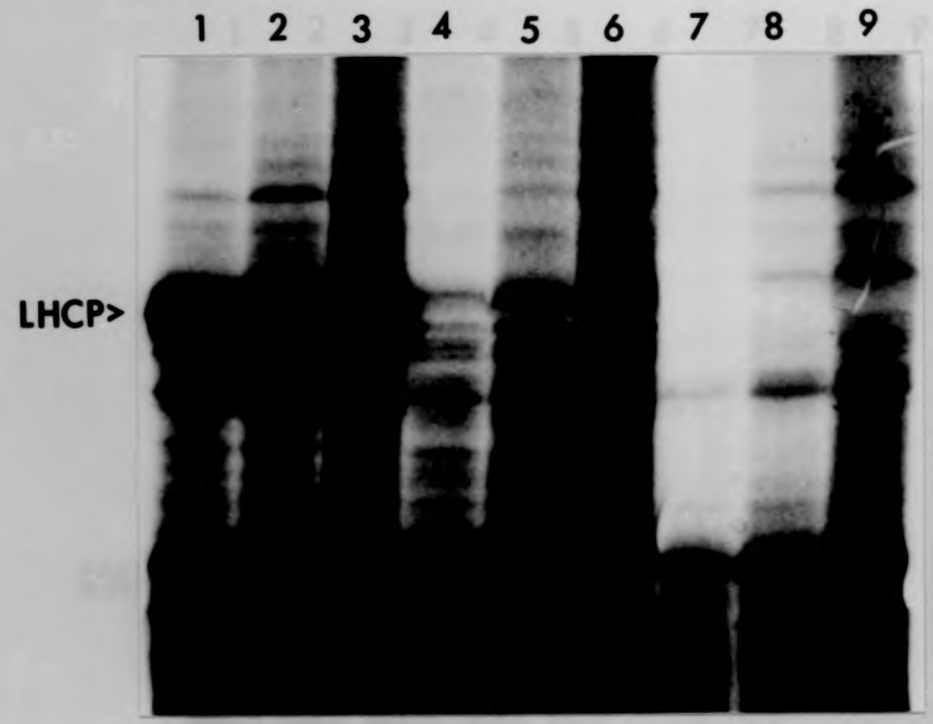
Track 1 : control incubation - 8 min
Track 2 : control incubation - 20 min
Track 3 : control incubation - 40 min
Track 4 : Thialysine incorporation - 8 min
Track 5 : Thialysine incorporation - 20 min
Track 6 : Thialysine incorporation - 40 min
Track 7 : Azetidine carboxylate incorporation - 8 min
Track 8 : Azetidine carboxylate incorporation - 20 min
Track 9 : Azetidine carboxylate incorporation - 40 min

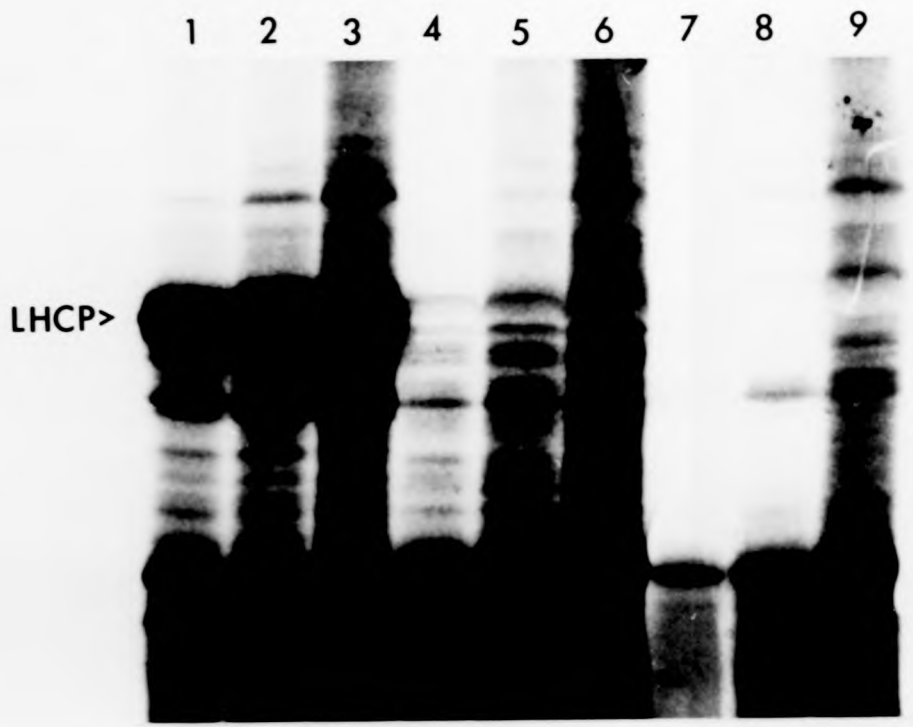


B



B


1
2
3
4
5
6
7
8
9
LHCP>


1 2 3 4 5 6 7 8 9
LHCP>

either amino acid analogue. However, as with the import of stromal polypeptides, the import of several thylakoid polypeptides is not apparently affected by analogue-substitution.

C. Discussion

The experiments described in this section show that *in vitro*-synthesised P20 is rapidly imported into isolated intact chloroplasts and processed to mature small subunit. None of the experimental conditions have led to the appearance of P20 inside the chloroplasts, suggesting that processing may be an integral part of the transport mechanism. If this is so, the results with iodoacetate-treated P20 show that the second cleavage reaction is not required for import into the organelle; carboxymethylated P20 is taken up and converted to a number of polypeptides of molecular weight 18,000 and below. The most likely explanation for the number of bands observed is that carboxymethylated P20 is taken up and converted to the P18 form, and that in the absence of further processing, this polypeptide is partially degraded inside the chloroplast. The P18 polypeptide may be particularly susceptible to non-specific proteolysis because it cannot be assembled into carboxylase holoenzyme; Schmidt and Mishkind (1983) have shown that unassembled small subunits are rapidly degraded in chloroplasts of *Chlamydomonas reinhardii*.

While the results shown in Fig. 27 are consistent with the possibility that processing is an essential step in the uptake, other interpretations of the data are possible. The metal-chelators, 1,10-phenanthroline and bathophenanthroline disulphonate, will have many side-effects on chloroplast function, a number of which could influence the import machinery. For example, many of the components of the photosynthetic machinery are metalloproteins.

The low rate of import of analogue-substituted P20 can also be explained in terms other than the inhibition of processing. It is highly likely that the chloroplast envelope contains receptors which specifically recognise precursors destined for import; the substitution of proline, arginine or lysine residues in the P20 chain may prevent recognition of the abnormal precursors by these import receptors. It is even possible that the import receptors and the processing enzyme recognise the same features of the precursor.

Similarly, the observed inhibition of import of other chloroplast polypeptides (Fig. 30) could be due either to poor recognition of the abnormal polypeptides by the import receptors or to inhibition of processing of the putative precursors. However, a significant finding is that the import of several polypeptides is not affected by incorporation of amino acid analogues. This result suggests that lysine and proline residues in these polypeptides do not play a significant role in the targeting of the import receptors or processing enzyme. A prediction from this finding is that the extension sequences of imported precursor polypeptides may differ markedly with respect to the functional significance of prolyl and lysyl residues. Again, further sequence analysis of precursors is required before the information specified by the extension sequence can be studied in detail.

SECTION IV - GENERAL DISCUSSION

1. PURIFICATION AND CHARACTERISATION OF SMALL SUBUNIT PRECURSOR PROCESSING ACTIVITY

The date presented in this thesis suggest that a single, high molecular weight metalloprotease is responsible for the processing of P20 and pre-plastocyanin to their mature sizes in pea chloroplasts. The stromal processing activity has been extensively purified such that the most highly purified fractions exhibit only 6-10 bands on a silver-stained SDS-polyacrylamide gel, including those representing the large and small subunits of ribulose bisphosphate carboxylase. Further purification is required to assign one or more of these bands to the processing enzyme.

A number of laboratories have reported the presence of proteolytic activities in chloroplast extracts. Chloroplast proteins such as the large subunit of ribulose bisphosphate carboxylase, glutamate synthase, and cytochrome c have been shown to be partially degraded during extraction from several plants (Gray and Kekwick, 1974; Gray, 1980; Wallsgrove et al., 1977). Furthermore, it is likely that chloroplasts contain all four categories of protease described in Section I.4, since marker inhibitors of each category have been shown to inhibit proteases in leaf extracts. Phenylmethylsulphonyl fluoride (PMSF), mercurial compounds (which inhibit thiol proteases), 1,10-phenanthroline and pepstatin (an inhibitor of acid proteases) have all been effective in reducing proteolytic activity in leaf extracts (Frith et al., 1978; Wittenbach, 1978; Ragster and Chrispeels, 1979; Takahashi et al., 1974).

Breakdown of ribulose bisphosphate carboxylase has been studied in several laboratories, and a wide range of chloroplast endoproteases have been shown to carry out hydroylsis *in vitro*. A feature common to most of the enzymes is a pH optimum of 4 to 5 (Peoples and Dalling, 1978; Wittenbach, 1978; Peoples *et al*., 1979; Miller and Huffaker, 1982; Thomas and Huffaker, 1981). In another study, Dalling *et al*. (1983) reported the isolation of two distinct peptide hydrolase activities from barley chloroplasts, with pH optima of 4.5 and 6.5.

A highly active and selective chloroplast proteolytic activity has been reported by Schmidt and Mishkind (1983), who studied the import of ribulose bisphosphate carboxylase small subunit into chloroplasts of *Chlamydomonas* which have been depleted of pools of the large subunit. These workers found that the newly imported small subunits were rapidly, and apparently selectively degraded, and proposed that the small subunits were particularly susceptible to proteolysis in the absence of assembly into the holoenzyme. Characterisation of the protease(s) responsible could not be carried out because no activity was exhibited by cell extracts.

It is clear from the above reports that chloroplasts contain a number of proteolytic enzymes, many of which are capable of carrying out hydrolysis *in vitro* when extracted from the intact organelle. Few of these proteases have been intensively studied, but it is likely that the majority are non-specific proteases such as those described in Section I.4A-D, capable of hydrolysing a wide variety of polypeptide substrates (including non-chloroplast proteins). Data presented in this thesis, SectionIII.1-4, have described the partial purification and

characterisation of a chloroplast protease using as an assay the cleavage of P20 to yield the mature size small subunit. It is proposed that this protease is responsible for the processing of precursors imported into the chloroplast, and that this reaction is carried out with a high degree of specificity. The evidence for this proposal comes from the following observations:

1) The enzyme processes P20 and pre-plastocyanin to the mature size as judged by SDS-polyacrylamide gel electrophoresis.

2) No activity is displayed against the mature forms of the above polypeptides.

3) The enzyme is inactive against SV40 and rotavirus mRNA translation products, or endogenous translation products of the wheat-germ system.

4) Highly purified preparations of processing enzyme can be stored at $4^{o}C$ for at least a week with no significant loss of activity, indicating that no autocatalytic degradation takes place. Furthermore, during the same period of storage there is no apparent degradation of contaminating proteins in the preparation (as judged by SDS polyacrylamide gel electrophoresis).

5) Substitution of proline, arginine or lysine residues in the P20 chain during translation renders the abnormal precursors virtually immune to processing by the partially purified enzyme. This finding suggests that the enzyme is not a non-specific protease such as those described in Section I.4A-D, since such proteases usually

carry out hydrolysis after binding to the side-chain of a particular amino acid or family of amino acids. For example, if substitution of lysine/arginine residues produced the observed inhibition of processing, it would be possible that the purified protease was a trypsin-like enzyme which cleaves proteins at positively-charged residues. This cannot, however, be the case, since substitution of proline residues also produces the observed inhibition. Such a reaction specificity is in any case unlikely since P20 contains a number of proline, argine and lysine residues, but is only cleaved to the mature size by the purified protease.

While the data presented in this thesis suggest that a highly specific protease carries out the processing of P20 and pre-plastocyanin, the results do not indicate whether the protease is responsible for the processing of all precursors imported from the cytoplasm; a greater number of precursors must first be tested with the purified processing enzyme.

Prior to the onset of this work, it was considered possible that each compartment of the chloroplast may contain a different processing enzyme which specifically recognised precursors destined for that compartment. The finding that the isolated processing enzyme cleaves pre-plastocyanin to the mature size suggests that both stromal and thylakoidal protein precursors are processed by the same enzyme. It is not known how two soluble proteins are directed to different, membrane-bound compartments even though their precursor forms are apparently cleaved by the same enzyme. It will be of great interest to determine whether the two precursors are imported by the same transport system, or whether

different systems are involved in the transport of proteins to the various compartments.

A particularly important line of approach will be to study the synthesis and import of the proteins present in the two chloroplast envelope membranes. Flugge and Wessel (1984) have shown that three envelope proteins are synthesised on cytoplasmic ribosomes as larger precursors. It will be interesting to determine whether the precursors are processed to the mature size by the enzyme partially purified in this study. If so, the precursors must become temporarily accessible to the stromal compartment in order for processing to take place if, as is believed, the enzyme is located in the stroma. Such a processing mechanims has been demonstrated for the maturation of some precursors imported into yeast mitochondria. The inter-membrane space enzymes cytochrome b_2 and cytochrome C_1 are imported, at least partially, into the matrix where they are cleaved by a processing enzyme similar to the one isolated in this study (Daum et al., 1982; Ohashi et al., 1928). An interesting feature of the maturation of these proteins is that they are processed in two steps; the matrix-localised processing enzyme cleaves the precursors to an intermediate form, which is then processed to the mature size by a second processing enzyme thought to be located outside the matrix. The import of proteins into the mitochondrial outer membrane is thought to proceed by a different mechanism which does not involve proteolytic processing (Freitag et al., 1982; Gasser and Schatz, in press). It remains to be seen whether chloroplast envelope membrane proteins are imported by similar mechanisms.

The matrix-localised processing enzyme in yeast mitochondria has been

partially purified by two groups (Bohni et al., 1983; McAda and Douglas, 1982) and shows some similarity to the enzyme described in this study. The mitochondrial enzyme has been shown to be a highly specific metalloprotease of high molecular weight (about 115,000 compared to 180,000 for the chloroplast enzyme).

The finding that processing of P20 to the mature size proceeds via a processing intermediate provides strong evidence that the processing enzyme is an endoprotease which carries out two successive cleavages. However, in none of the processing experiments performed in this study have any peptides been observed which could represent processed fragments, even in experiments carried out with care taken not to run small peptides off the end of the gel. The same result was obtained by Smith (1980) who studied the processing of P20 by intact isolated chloroplasts and crude stromal extracts. Possibly the small size of the fragments (2,000 and 4,000 mol.wt.) precludes resolution by the SDS-polyacrylamide gel electrophoresis system used in this study. Dobberstein et al. (1977) studied the processing of Chlamydomonas small subunit precursor by a soluble cell lysate, and suggested that a single endoproteolytic cleavage was involved since they detected a peptide fragment large enough to account for the processed piece. However, no further information about this fragment has been published.

2. THE MECHANISM AND ROLE OF THE PROCESSING OF IMPORTED CHLOROPLAST PRECURSOR POLYPEPTIDES

The experiments described in Sections II.4 and III.5 suggest that the processing of P20 takes place by a mechanism fundamentally different to that of the majority of proteolytic cleavages carried out by "general" proteases. The data indicate that a single enzyme probably cleaves P20 in two steps, despite the presence of different residues at the two cleavage sites, showing that the proteolytic events are not initiated by the binding of the enzyme to a residue involved in each scissile bond.

The data presented in Section III.4A represent the first report of the two-step processing of a cytoplasmically-synthesised chloroplast precursor polypeptide, but since only a very small number of precursors have been studied, it is not possible to state whether this mode of processing is unusual or widespread. Neither is it known whether carboxylase small subunit precursors from other plant species are processed in two steps.

The site of the first cleavage in the processing of P20 is not known, and it is recommended that in future work this be deduced, since the basis for the specificity of the processing enzyme may then become more apparent. This could be achieved by synthesis of P20 in the presence of a number of labelled amino acids in addition to [^{35}S]-methionine, followed by incubation of the labelled precursor, first with iodoacetate, and then with processing enzyme. This procedure should

result in the production of the P18 intermediate. The site of the first cleavage site could then be determined by sequential Edman degradation of the polypeptide after excision of the band from the dried gel; the positions of the labelled amino acids in the P18 sequence could then be compared with the positions of the amino acids in the known extension sequence of P20 (Cashmore, 1983) to deduce the site of cleavage.

The finding that a single enzyme may be responsible for both cleavages raises an important question: is cleavage at the first site a prerequisite for cleavage at the second site? It has been suggested earlier that the features of the precursor that are recognised by the processing enzyme may be duplicated, i.e. the processing enzyme may recognise and cleave both scissile bonds in an identical manner. If this is the case, the processing enzyme may be capable of cleaving at the second site while the first cleavage site is still intact. Alternatively, the conformation of the extension sequence may be such that the second cleavage site is masked from the processing enzyme until the first cleavage has taken place. Another possibility is that the second cleavage site in intact P20 is physically accessible to the processing enzyme, but is not recognised by the processing enzyme until cleavage at the first site has taken place, i.e. that the first cleavage causes a conformational change such that the second cleavage site becomes "processable". Further studies are required to resolve these possibilities. In particular, further purification of the processing enzyme should be attempted, in order to confirm the belief that a single enzyme carries out both cleavages.

The import of proteins into mitochondria has been studied in detail by a

number of laboratories (for a review see Schatz and Butow, 1983). Most of the imported proteins are initially synthesised as larger precursors which are processed to the mature size by a matrix-localised protease, apparently by a single cleavage. However, a smaller number of precursors have been shown to undergo two-step processing (Daum et al., 1982; Ohashi et al., 1982; Teintze et al., 1982). The significance of the two-step processing reactions is not understood; similarly, this author can offer no rationalisation of the two-step processing of small subunit precursor.

It has not been possible to determine the basis for the specificity of the processing enzyme, but the data described in Section III.4 provide some potentially significant clues. The inhibition of the second cleavage by carboxymethylation of the precursor suggests that the processing enzyme may be sterically hindered by the formation of a large side-chain on the cysteine residue at the second cleavage site. However, though the size of the side-chains of the residues (or at least one residue) of the scissile bonds may be constrained, the identity does not seem to be significant since different residues are clearly present at the two cleavage sites. Furthermore, the side chains of the residues involved in the scissile bonds can, and do, have different charge characteristics; at the pH optimum for processing of P20 (9.0) the cysteine thiol group is predominantly negatively charged (pK_a = 8.2). Neither of the residues involved at the first cleavage site can be negatively charged, because the extension sequence contains only one other negatively-charged residue (an aspartate) and this residue is close to the second cleavage site (see Appendix).

The inhibition of processing by analogue-substitution of P20 (Section III.4B) is consistent with the suggestion that prolyl and positively-charged residues in the extension sequences of small subunit precursors, play an important role in targeting the processing enzyme. In particular, the results obtained with azetidine carboxylate suggest that correct conformation of the precursor, either in the extension sequence, the mature sequence or both, is essential for efficient recognition by the processing enzyme. The results obtained with canavanine and thialysine suggest that arginyl and lysyl residues may be more directly involved in the precursor-processing enzyme interaction. However, the inhibition caused by incorporation of these analogues may also stem from alteration of three-dimensional structure in the precursor.

It is apparent that the interpretation of the results obtained with amino acid analogues is complicated by the distribution of the substituted residues (proline, lysine and arginine) throughout the entire precursor molecule. Clearly, more meaningful results might be obtained if processing of P20 could be inhibited by the incorporation of amino acid analogues into specific areas of the precursor. Analysis of the primary sequence of P20 shows that the mature small subunit sequence contains no asparagine residues (Takruri et al., 1981), but that the extension sequence contains two such residues near the second cleavage site (see Appendix). Useful information about the structural requirements of the processing site might be obtained bysubstitution of these asparagine residues by an amino acid analogue. A candidate for this approach is the analogue DL-threo-B-fluoroasparagine. Hortin et al. (1983) have shown that this compound is incorporated into protein in cell-free translation systems. This analogue is not commercially

available, but can be synthesised by the method of Stern *et al.* (1982).

Other useful information about the basis for the specificity of the processing reaction might be obtained by assaying the purified enzyme for the processing of P20 which had been structurally altered in the mature small subunit part of the sequence. At present it is not known if the mature small subunit section of the precursor plays a part in targeting the processing enzyme. It is possible that changes in the conformation of the mature section of the precursor would affect the conformation of the extension sequence and thereby inhibit processing. This possibility could be tested by analogue substitution or covalent modification of P20 residues that occur in the mature small subunit sequence but not in the extension sequence. Such residues include tryptophan, tyrosine, glutamic acid and histidine.

The other major unsolved question relates to the role of the processing enzyme in the chloroplast: is processing an integral part of the transport mechanism? The characterisation of the processing reaction *in vitro*, described in Sections III.1 to III.4, include the identification of several inhibitors of the purified processing enzyme. These inhibitors were tested for their effect on uptake of P20 by isolated chloroplasts (Section III.5), and were found to inhibit import markedly. Unfortunately, for the reasons cited in Section III.5C, the inhibition of import could be explained in terms other than the inhibition of the processing enzyme *in situ*. The crucial experiment will be to demonstrate the presence of imported, intact precursor molecules inside intact chloroplasts; this has not yet been achieved either in this study or in any other reports. One possible approach is to test for

available, but can be synthesised by the method of Stern *et al.* (1982).

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other methods of inhibiting the processing reaction, for example by incorporation of other amino acid analogues into P20 or by modification of P20 with a number of available protein modification reagents. The inhibitor(s) could then be included in uptake assays as in Section III.5B.

While the role, if any, of processing in the transport of P20 into chloroplsts remains obscure, the demonstration of the import of iodoacetate-treated P20 into intact chloroplasts (Section III.5B) strongly suggests that the second cleavage cannot be involved. The problem is therefore reduced to a consideration of the role of the first cleavage in the transport mechanism.

When attempting to define the sequence of events involved in the import of proteins into chloroplasts, it must be emphasised that the in vitro reconstitution systems such as that described in Section III.5 and in other published studies, have not been fully characterised. It is not yet possible to state that the import of P20 results solely from an interaction of the precursor molecule with the intact chloroplast, because it is possible that factors present in the wheat-germ extract may be involved in the in vitro uptake of the precursors. Recent studies on the import of proteins by isolated mitochondria suggest that such factors play an important role in the overall import process. Miura et al. (1983) have shown that the in vitro-synthesised precursor of rat liver ornithine carbamoyltransferase is taken up and processed to the mature size by isolated rat liver mitochondria. In this study the precursor was synthesised using a rabbit reticulocyte lysate protein-synthesising system rather than the wheat-germ system. These workers

found that the import of the precursor was markedly stimulated by the addition of dialysed reticulocyte lysate extract to the uptake incubation mixture (which consisted of reticulocyte lysate extract containing labelled precursor, together with isolated mitochondria). The stimulatory effect of the added dialysed extract was completely lost by trypsin treatment or heat treatment at 100°C for 2 min, suggesting that the factor(s) was a protein(s).

In another study, Argan *et al.* (1983) measured the import and processing of the above precursor by rat heart mitochondria. The precursor was again synthesised *in vitro* using the rabbit reticulocyte lysate system. These workers chromatographed the reticulocyte translation mixture, containing newly synthesised precursor, on a Sephadex G-25 column and found that the precursor, which was eluted in the void volume, was no longer imported into isolated mitochondria. This result indicates that an essential component of molecular mass below 5000 daltons was absent from the uptake incubation mixture. The component was found to derive from the reticulocyte lysate itself rather than from other compounds added to the incubation mixture, since addition of reticulocyte lysate to the Sephadex G-25 eluate restored uptake, but addition of the other ingredients, either singly or in combination, did not.

Taken together, the results from these studies indicate that factors from the reticulocyte lysate, including a protein(s) and possibly other smaller molecules, are involved in the import of precursors by isolated mitochondria. The same factors may well be involved in the *in vivo* biogenesis of mitochondria. Similarly, it is possible that components of the wheat-germ extract are directly involved in the *in vitro* uptake

of precursors by isolated chloroplasts, and that, by extension, cytosolic factors are involved in the *in vivo* import of precursors by chloroplasts.

Many of the approaches that have been used to study the transport of proteins by chloroplasts have been made difficult by the fact that only minute quantities of precursor can be synthesised *in vitro*. No P20 can be detected in lysates of leaf cells, indicating that the precursor is rapidly imported into chloroplasts *in vivo*. This problem might be alleviated by further charcterisation of the processing reaction aimed at the development of a procesing inhibitor which could be used to inhibit the processing of P20 in whole plants. The data in Section III.4B suggest one approach: the use of amino acid analogues to render P20 immune to processing. Pea leaf cell contents can be labelled to high specific activity by "painting" [^{35}S]-methionine onto the surface of the leaves. The label is readily taken into the leaves and incorporated into protein. Equally, it may be possible to introduce amino acid analogue into leaf cell proteins in sufficient quantity to cause the accumulation of chemical amounts of unprocessable P20.

By way of comparison, it should be noted that several of these problems have been solved in similar studies of protein transport into mitochondria. Reid and Schatz (1982) have been able to purify chemical amounts of F1-ATPase b-subunit precursor by growing yeast cells in the presence of an uncoupler of oxidative phosphorylation. The presence of the uncoupler strongly inhibits the import of proteins into mitochondria (discussed in Section I.4E).

Recent evidence suggests that the import of proteins into mitochondria can proceed in the absence of processing. Zwizinski and Neupert (1983) have reported that two precursors of mitochondrial proteins are imported but not processed in the presence of EDTA or 1,10-phenanthroline. Further experiments are required to determine whether the import of proteins into chloroplasts can take place in the absence of processing.

APPENDIX

APPENDIX

1. Extension Sequences for the Small Subunit of RuBPCase

Pea	NH_2 - M A S M I S S S A V T T V S R A S R G Q S A A
Soybean	NH_2 - M A S S M I S S P A V T T V N R A G A G M
Wheat pW9	NH_2 - M A P A V M A S S A T T

Pea	V A P F G G L K S M T G F P V - K K V N T D I
Soybean	V A P F T G L K S M A G F P T - R K T N N D I
Wheat pW9	V A P F Q G L K S T A G L P I S C R S G S T G

Pea	T S I T S N G G R V K C | M Q V W P P I
Soybean	T S I A S N G G R V Q C | M Q V W P P I
Wheat pW9	L S S V S N G G R I R C | M Q V W P I E

Transit Peptide | Mature Small Subunit

(Cashmore, 1983; Berry-Lowe *et al.*, 1982; Coruzzi *et al.*, 1983).

The one-letter code for amino acids is given overleaf.

2. One-letter code for amino acids

A	Alanine
C	Cysteine
D	Aspartate
E	Glutamate
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

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